AWARD NUMBER: W81XWH-14-1-0153

TITLE: Identification of Novel Drug Targets and Lead Compounds for Advanced Prostate Cancer through Genomic and Cheminformatic Analyses

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REPORT DATE: October 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Final	01 Aug 2014 - 31 Jul 2016

4. TITLE AND SUBTITLE
Identification of Novel Drug Targets and Lead Compounds for Advanced Prostate Cancer through Genomic and Cheminformatic Analyses

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0153

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Prostate cancer (PCa) is one of the most common cancers for males in the United States and Canada. When PCa is diagnosed early, it can be treated by surgery or radiation. However, in about 30% of cases when PCa recurs or metastasizes, the disease is managed primarily by hormone therapy. Unfortunately, the effectiveness of this therapy is temporary due to emerging resistance mechanisms. Thus, the limited effectiveness and side effects of current PCa treatments demand for new therapeutics to treat advanced PCa. With the accumulated publicly available genomic data and recent advancements in bioinformatic and cheminformatic algorithms, this research project aims to apply comprehensive analyses of cancer genomes and transcriptomes to identify new drug targets, followed by the discovery of lead compounds through virtual screening techniques and biological assays. The outcome of this project can lead to new generations of anti-PCa drugs to treat cases where the cancer cells have developed resistance to existing drugs, by targeting other alternative pathways.

15. SUBJECT TERMS
Prostate cancer, genomics, transcriptomics, proteomics, cheminformatics, computer-aided drug design, small molecule inhibitors, biological assays, compound validation.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	50	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION

Prostate cancer (PCa) is one of the most common cancers for males in the United States and Canada, with about 245,000 cases and 32,000 deaths in 2015[1,2]. When PCa is diagnosed early, it can often be treated by surgery or radiation. However, in about 30% of cases when PCa recurs and/or metastasizes, the disease is managed primarily by the hormone therapy that reduces the level of androgens or blocks their binding to the androgen receptor (AR). The effectiveness of this therapy is temporary due to emerging resistance mechanisms related to AR. While several chemo- and immunotherapies are available for managing such advanced PCa, they offer only modest survival benefits. Because of the limited effectiveness and significant side effects of current PCa treatments, there is a pressing demand for new therapeutics to be developed to treat aggressive and resistant prostate cancer. With the accumulated publicly available genomic data and recent advancements in bioinformatic and cheminformatic algorithms, this research project aims to apply comprehensive analyses of cancer genomes and transcriptomes to identify new drug targets, followed by the discovery of hit compounds through virtual screening techniques and biological assays. The outcome of this project can lead to new generations of anti-PCa drugs to treat cases where the cancer cells have developed resistance to existing drugs, by targeting other alternative pathways.

2. KEYWORDS

Prostate cancer
Metastatic castration-resistant prostate cancer
Cancer-specific aberrations
Genomics
Transcriptomics
Proteomics
Bioinformatics
Cheminformatics
Computer-aided drug design
Small molecule inhibitors
In silico compound screening
Compound validation by biological assays

3. ACCOMPLISHMENTS

What were the major goals of the project?

Year 1 (August 1, 2014 to July 31, 2015), completed

Specific Aim 1 - Genomics: identification of prostate cancer-related aberrations in gene regulation.	Proposed dates (month/year)	Actual completion dates (month/year)
Major Goal 1: Collect available genomic data from prostate tumor and normal cells.	Aug/2014 - Nov/2014	Aug/2014 - Nov/2014
Milestone 1: Construction of a shared and central database of genomic data for prostate cancer research at Vancouver Prostate Centre, University of British Columbia	Nov/2014	Nov/2014
Major Goal 2: Analyze the genomic data to identify prostate cancer-specific aberrations.	Dec/2014 - May/2015	Dec/2014 - May/2015
Major Goal 3: Identify new drug targets in prostate cancer.	June/2015 - July/2015	June/2015 - July/2015
Milestone 2: Identification of three drug targets in prostate cancer.	July/2015	July/2015
Milestone 3: One co-author manuscript on bioinformatic analyses of genomic data and drug target identification in prostate cancer.	July/2015	Dec/2014

Year 2 (August 1, 2015 to July 31, 2016), completed

Specific Aim 2 - Cheminformatics: development of new classes of anti-prostate cancer drugs.	Proposed dates (month/year)	Actual completion dates (month/year)
Major Goal 4: Construct protein structural models and identify potential drug binding sites for each of three targets.	Aug/2015 - Nov/2015	Aug/2015 - Nov/2015
Milestone 4: Determine binding pockets of each of the three targets for subsequent virtual screening.	Nov/2015	Nov/2015
Major Goal 5: Virtual compound screening against the binding pocket of drug target #1.	Dec/2015 - Feb/2016	Dec/2015 - Feb/2016
Milestone 5: Identify 200 compounds with high binding potential against drug target #1 for further biological validation.	Feb/2016	Feb/2016
Major Goal 6: Virtual compound screening against the binding pocket of drug target #2.	Feb/2016 - Apr/2016	Feb/2016 - Apr/2016

Milestone 6: Identify 200 compounds with high binding potential against drug target #2 for further biological validation.	Apr/2016	Apr/2016
Major Goal 7: Virtual compound screening against the binding pocket of drug target #3.	Apr/2016 - June/2016	Apr/2016 - June/2016
Milestone 7: Identify 200 compounds with high binding potential against drug target #3 for further biological validation.	June/2016	June/2016
Major Goal 8: Compound validations by biological assays.	Aug/2015 - July/2016	Aug/2015 - July/2016
Milestone 8: Identify lead compounds with high biological activity against drug target #1, #2 and #3.	June/2016 - July/2016	June/2016 - July/2016
Milestone 9: Up to three co-author manuscripts on lead compounds against drug target #1, #2 and #3.	July/2016	July/2016

What was accomplished under these goals?

As outlined in the previous section and in the approved SOW, the three major goals for the Specific Aim 1, "Genomics: identification of prostate cancer-related aberrations in gene regulation", have been accomplished during the **Year 1** period (August 1, 2014 to July 31, 2015). The three accomplished goals are: **1)** collect available genomic data from prostate tumor and normal cells; **2)** analyze the genomic data to identify prostate cancer-specific aberrations; and **3)** identify new drug targets in prostate cancer.

As also outlined in the previous section and in the approved SOW, the five major goals for the Specific Aim 2, "Cheminformatics: development of new classes of anti-prostate cancer drugs", have been accomplished during the **Year 2** period (August 1, 2015 to July 31, 2016). The five accomplished goals are: **4)** construct protein structural models and identify potential drug binding sites for each of three targets; **5)** virtual compound screening against the binding pocket of drug target #1; **6)** virtual compound screening against the binding pocket of drug target #2; **7)** virtual compound screening against the binding pocket of drug target #3; and **8)** compound validations by biological assays.

Major Goal 1: Collect available genomic data from prostate tumor and normal cells.

1) Major activities

Publicly available PCa genomic and transcriptomic datasets from high-throughput experiments have been collected from a number of sources including the Gene Expression Omnibus (GEO)[3] and The Cancer Genome Atlas (TCGA)[4]. The public available datasets were downloaded through the corresponding web portals. In collaboration with Dr. Anna Lapuk, in-house high-quality datasets previously derived from the patient cohort at the Vancouver Prostate Centre (VPC), University of British Columbia (UBC) have been collected. Both the public and VPC datasets have

been stored in a centralized hard drive on the shared VPC network. All the datasets have been checked for data quality and consistency (with respect to their original publications). These data files have been converted and stored as the following three formats: 1) tab-delimited text files for easy importing to other analysis programs; 2) data tables in the MySQL database for fast data searching; and 3) data objects in the R computational environment for all the subsequent statistical analyses. A number of scripts based on the PERL programming language have been created for data processing and cross-referencing among the difference datasets.

2) Specific objectives

The main objective of Major Goal 1 is to create a comprehensive collection of publicly and internally available PCa genomic and transcriptomic datasets not only for the analyses performed in this project, but also for future usages by other colleagues at the VPC, UBC.

3) Significant results/key outcomes

As shown in **Table 1**, this comprehensive collection of datasets consists of 2 DNA-Seq, 5 RNA-Seq, 12 microarrays and 6 ChIP-Seq datasets from a total of 1435 samples, including multiple prostate cancer cell lines. In particular, this collection also contains a high-quality in-house dataset with matched DNA-Seq and RNA-Seq data from 26 prostate tumours and 5 normal prostate samples from the patients[5].

Table 1. Prostate cancer (PCa) genomic and transcriptomic datasets collected.

Source/Accession	Data type	Total number of samples	Sample type	PubMed ID
TCGA	DNA-Seq, RNA-Seq, SNP array	550	Patient samples	
GEO/GSE38674	DNA-Seq	2	LnCaP	23245995
VPC	RNA-Seq	31	Patient samples	25155515
GEO/GSE14092	RNA-Seq	3	LNCaP	20478527
	RNA-Seq	42	Patient samples	22389870
GEO/GSE34780	RNA-Seq	6	LNCaP	23034120
GEO/GSE38676	RNA-Seq	2	LNCaP	23245995
MSKCC/GEO/GSE21034	microarray	370	Patient samples	20579941
GEO/GSE60771	microarray	16	VCaP	25263440
GEO/GSE58940	microarray	4	VCaP	
GEO/GSE53994	microarray	15	VCaP	24591637
GEO/GSE15091	microarray	7	PC3	19668381
GEO/GSE14028	microarray	15	LNCaP, VCaP	20478527
GEO/GSE28948, GSE35540	microarray	12	VCaP	22531786

GEO/GSE36549	microarray	14	LNCaP, VCaP	22710436
GEO/GSE15805	microarray	155	LNCaP	22955617
GEO/GSE28680	microarray	24	Patient samples	23260764
GEO/GSE39654	microarray	16	LNCaP-1F5, VCaP	23269278
GEO/GSE11428	microarray	21	LnCaP	
GEO/GSE14092	ChIP-Seq	57	LNCaP, VCaP, patient samples	20478527
GEO/GSE20042	ChIP-Seq	5	LNCaP	20208536
GEO/GSE28950	ChIP-Seq	15	VCaP	22531786
GEO/GSE38682, GSE38683, GSE38684	ChIP-Seq	6	LNCaP	23245995
GEO/GSE28219	ChIP-Seq	25	LNCaP, VCaP, patient samples	23260764
GEO/GSE39879	ChIP-Seq	22	LNCaP-1F5, VCaP	23269278

Major Goal 2: Analyze the genomic data to identify prostate cancer-specific aberrations.

1) Major activities

In order to identify PCa-specific aberrations, the datasets collected from Major Goal 1 have been analyzed by using the following computational algorithms. Single nucleotide variants derived from the DNA-Seq data have been annotated by the gSearch program[6], and copy number variations were identified by the use of BIC-seq[7]. Gene fusion events have been identified by using DeFuse[8] on the RNA-Seq data. Differentially expressed genes from the microarray and RNA-seq data were determined by using t-test for two group comparison (tumor vs. normal). In particular, overexpressed genes were identified from each dataset by the following steps: 1) log2 transformation; 2) two sample t-test between tumour and normal samples; 3) multiple testing correction on p-values[9]; 4) selection of genes with corrected (adjusted) p-values < 0.05; and 5) among those with significant p-values, selection of genes with fold-change ≥ 2 (tumor vs. normal). Transcription factor binding data from ChIP-Seq have been analyzed by using the CompleteMOTIFs pipeline[10]. Additional analyses on the TCGA dataset were performed by using the computational tools available at cBioPortal[11].

2) Specific objectives

The main objective of Major Goal 2 is to perform comprehensive analyses on the different datasets (DNA-Seq, RNA-Seq, microarray, ChIP-Seq) collected previously from Major Goal 1. The results from these analyzes have been used to identify a list of candidate drug targets for prostate cancer (more details in Major Goal 3).

3) Significant results/key outcomes

The bioinformatic analyses performed on the TCGA prostate cancer dataset, consisted of 498 PCa patient tumour samples and 52 normal samples, have identified a list of frequent genomic alterations, as shown in **Table 2**. Among the top alterations are: gene fusions involving ERG,

ETV1 and ETV4; PTEN deletion; amplifications of genes including MYC, AR, and PIK3CA. The frequencies of these genomic alternations identified from the analyses is comparable to those reported in previous studies[12,13].

Table 2. Frequent genomic alterations in PCa patient samples (TCGA dataset).

Gene	Type of alterations	% of cases in the TCGA dataset
ERG	Gene fusion	46%
PTEN	Deletion	17%
NKX3-1	Deletion	17%
TMPRSS2	Deletion	16%
ETV1	Gene fusion	10%
TP53	Deletion	8%
MYC	Amplification	8%
AR	Amplification	7%
FOXA1	Amplification	6%
ETV4	Gene fusion	5%
PIK3CA	Amplification	5%

In addition to the genomic alterations listed above, gene expression analyses performed on the different datasets collected have revealed a number of genes that are overexpressed in PCa patient samples. **Figure 1** illustrates the results from gene expression analyses performed on the two largest datasets, MSKCC[14] and TCGA[4], as well as the in-house dataset from VPC[5]. Using a standard but stringent bioinformatic protocol, a total of 169, 24, 744 genes have been found to overexpress in the VPC, MSKCC and TCGA datasets, respectively (**Figure 1A**). While each dataset features a different list of genes, ERG is the only common gene overexpressed in all of the three datasets.

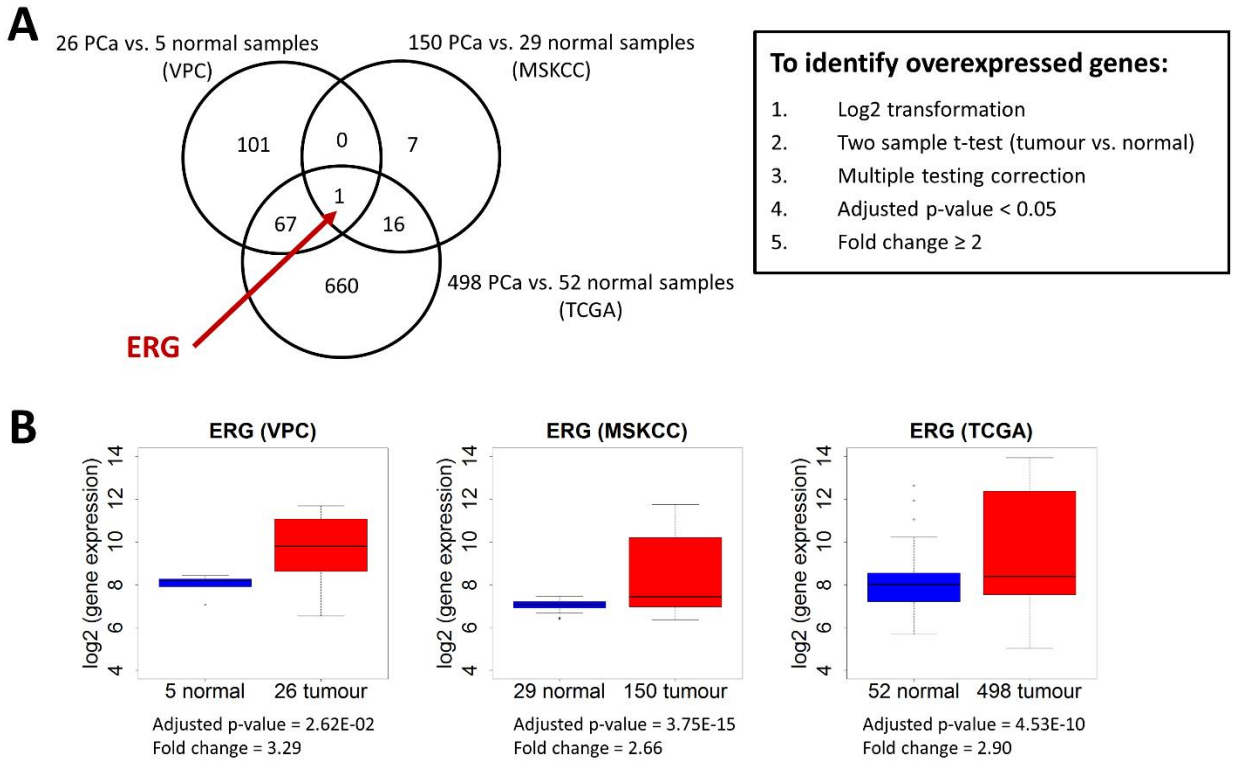


Figure 1. ERG is one of the most commonly overexpressed genes in PCa patient samples. A) A Venn diagram that shows the number of overexpressed (i.e. upregulated) genes from each of the three gene expression datasets: Vancouver Prostate Centre (VPC)[5], Memorial Sloan-Kettering Cancer Center (MSKCC)[14], and The Cancer Genome Atlas (TCGA)[4], based on a bioinformatic protocol as listed on the right panel. ERG is the only overexpressed gene from intersecting the three datasets. **B)** The fold changes of ERG gene expression in PCa tumour samples, compared to normal samples, range from 2.66 to 3.29.

To further explore other overexpressed genes (in addition to ERG), the above stringent bioinformatic protocol that required a gene to be overexpressed in all three datasets has been relaxed. **Figure 2** highlights other overexpressed genes in the TCGA dataset, which represents the largest PCa gene expression data currently available, in the three major gene families of interest: 1) ETS factors, 2) nuclear receptors, and 3) kinases. In addition, we have compared the gene expression profiles between samples with low ERG expression and those with high ERG expression within the total PCa dataset from TCGA (**Figure 2A**, bottom panel). Notably, ETV1 and ETV4 are overexpressed in PCa samples where ERG is expressed at lower level. This gene overexpression is likely due to the gene fusion involving ETV1 and ETV4, and it suggests that the different ETS factors (ERG, ETV1 and ETV4) may compensate the function of each other.

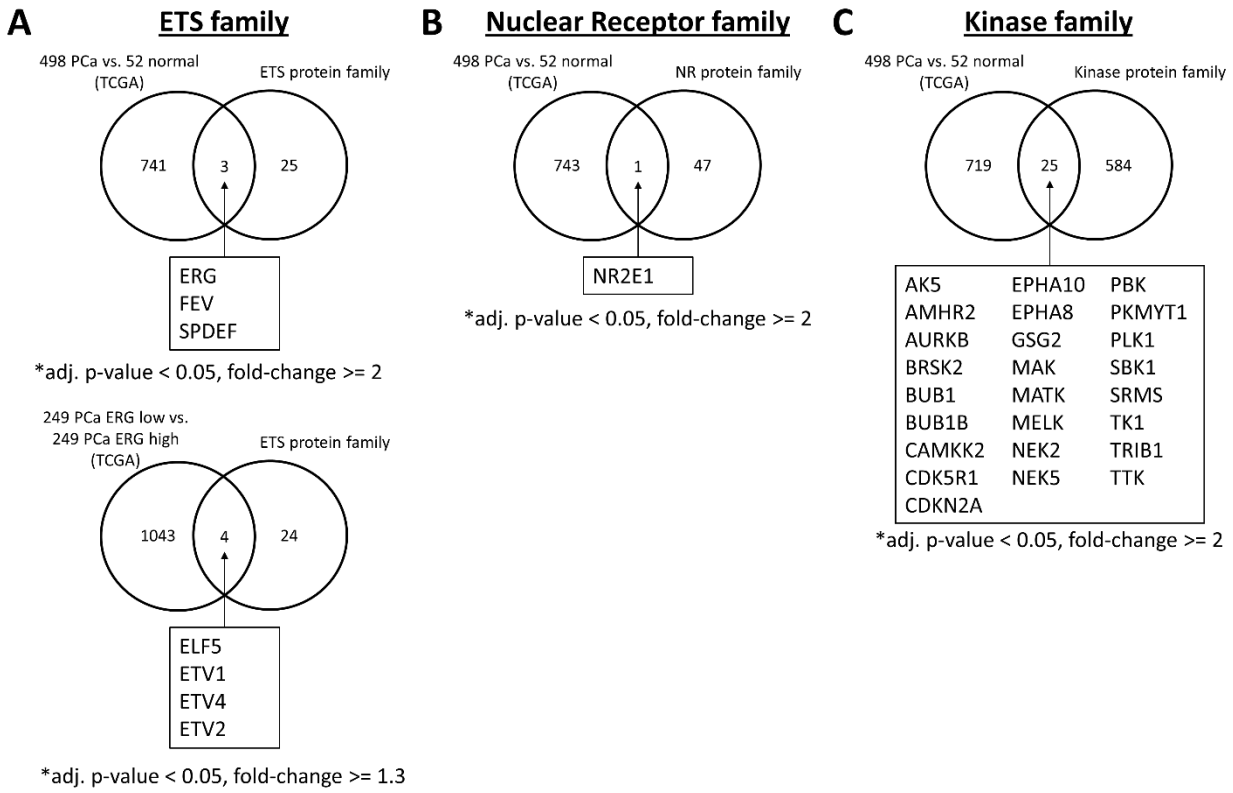
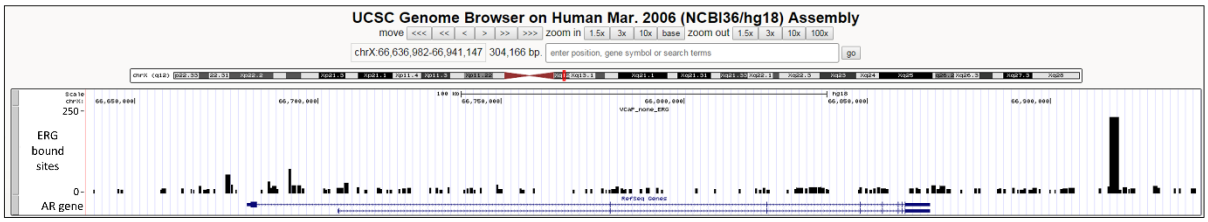


Figure 2. Overexpressed genes from the ETS, Nuclear Receptor (NR) and Kinase families in PCa patient samples. A) Top panel: A list of ETS genes (ERG, FEV, SPDEF) are overexpressed in the 498 PCa samples, compared to the 52 normal samples in the TCGA dataset. Bottom panel: Within the 498 PCa samples, 4 ETS genes are overexpressed in samples that have lower ERG expression. **B)** NR2E1 gene from the nuclear receptor family is overexpressed in the 498 PCa samples. **C)** A number of kinase genes are overexpressed in the 498 PCa smaples.

To gain more insights on DNA binding sites by the ERG transcription factor on chromosomes and nearby genes, several publicly available ChIP-Seq datasets have been analyzed. **Figure 3** features the result from analyzing one of the largest ChIP-Seq datasets[15], consisted of a total of 42,568 DNA sites bound by the ERG transcription factor in VCaP cells (a PCa cell line that harbours the ERG gene fusion and overexpresses the ERG protein). All of the 42,568 ERG-bound sites have been analyzed using the CompleteMOTIFs program[10], and **Figure 3B** shows the top nearby genes ranked by the number of ERG-bound sites. Interestingly, the gene Androgen Receptor (AR) is ranked as the second. Further visualization of the ERG-bound sites at the AR locus, using the UCSC Genome Browser[16], illustrated that the sites, with various amount of binding intensity (ranged from 0-250), span across the entire AR gene including both upstream and downstream regions (**Figure 3A**). This result suggests that overproduction of the ERG protein may have a significant influence on the AR transcription and the AR-regulated pathways in prostate cancer cells.

A



B

Entrez gene ID	Gene symbol	Chromosome	Transcription start	Transcription end	Number of ERG-bound sites (peaks)
8660	IRS2	chr13	109204185	109236915	133
367	AR	chrX	66705408	66860844	121
121793	C13orf16	chr13	110771016	110794595	83
60401	EDA2R	chrX	65732207	65752598	57
57507	ZNF608	chr5	124000509	124108704	44
6656	SOX1	chr13	111769914	111774021	40
352887	GYG2P1	chrY	13027923	13043397	38
23769	FLRT1	chr11	63627938	63643221	34
283487	C13orf29	chr13	110314335	110320656	31
55647	RAB20	chr13	109973414	110012072	29

Figure 3. Enrichment of ERG-bound sites at the Androgen Receptor (AR) gene locus based on ChIP-Seq analyses. A) Visualization of the intensity of ERG-bound sites (y-axis) at the AR gene locus (x-axis) on the UCSC Genome Browser. **B)** The top 10 genes, ranked by the number of nearby ERG-bound sites.

Major Goal 3: Identify new drug targets in prostate cancer.

1) Major activities

By analyzing the genomic and transcriptomic datasets as summarized in Major Goal 2, we have identified a number of target genes with aberrations such as genomic alterations and gene overexpression in patient PCa samples. In order to perform an initial assessment of structural availability and druggability on these potential targets, the protein sequence of each target was queried against the Protein Data Bank (PDB)[17] using Protein Blast[18]. Protein targets with resolved x-ray crystal or NMR structures were identified, and in other cases, those with homologous structural templates (≥ 70% sequence identity) suitable for subsequent homology modeling were determined. The program, Modeller v9.11 (with the 'allmodel' protocol)[19], was used to build 3D protein models for the potential targets, based on the structural templates identified from PDB. Then each protein model was subjected to the Site Finder algorithm, implemented in the Molecular Operating Environment (MOE) 2012, which used virtual atomic probes to search the protein surface for suitable small molecule binding pockets[20]. Each pocket was scored based on its size/deepness, solvent exposure, and the surrounding amino acid residues.

In addition to the two criteria as described above, PCa-specific aberrations and structural availability/druggability, a comprehensive literature has been performed on each potential target. In particular, we examined experimental results from previous studies that support 1) gain of cancerous phenotypes due to the overexpression of the target and/or 2) loss of cancerous features due to the knockdown of the target. All these information were presented to Dr. Paul Rennie (Mentor of this project), and together we have identified a total of three candidate drug targets that are suitable for Specific Aim 2 in Year 2, "Cheminformatics: development of new classes of anti-prostate cancer drugs".

2) Specific objectives

The main objective of Major Goal 3 is to narrow the list of potential drug targets down to three candidates with multiple lines of supporting evidence based on 1) PCa-specific aberrations as identified from Major Goal 2; 2) existing literature supporting the oncogenic functions of the drug targets; 3) expert knowledge from Dr. Rennie; and 4) structural availability and druggability. Preliminary protein modeling and initial binding site assessment have been performed on each of the drug target candidates. More comprehensive and in-depth structural evaluations based on advanced computational methods would be performed in Year 2 (Major Goal 4: Construct protein structural models and identify potential drug binding sites for each of three targets).

3) Significant results/key outcomes

Initial assessments on protein structures and druggability have been performed on a number of genes with PCa-specific aberrations, with a particular emphasis on the three gene families: ETS factors, nuclear receptors and kinases. **Figure 4** shows suitable drug-binding sites identified on the surfaces of ERG, ETV1 and ETV4 protein models. To further examine the similarity and difference among the three ETS protein sequences, a phylogenetic tree has been constructed using a multiple sequence alignment program, Clustal Omega[21]. As shown in **Figure 5**, the ETS family consists of a total of 28 members, and ERG and ETV1/4 are clustered to the ERG and PEA3 subfamilies, respectively. In addition, the sequence alignment indicates that ETV1/4 shares 61-65% of sequence identity with ERG; thus, it is feasible to develop specific small molecules targeting each of the three ETS factors by exploiting their sequence and structural differences.

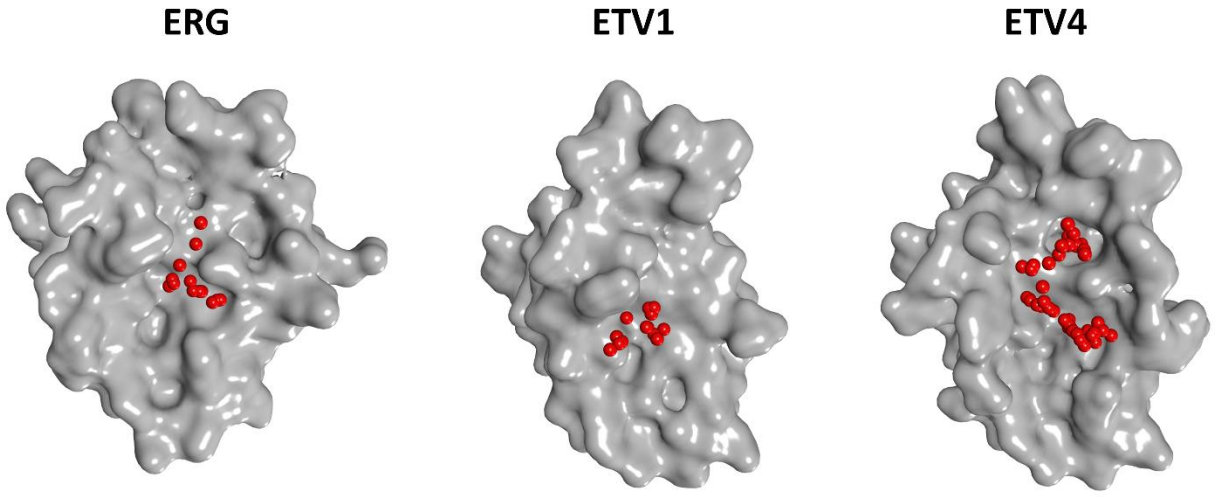


Figure 4. Initial assessment of druggable sites on the three drug target candidates. Surfaces of predicted protein models for the three drug target candidates: ERG, ETV1 and ETV4. Potential drug-binding sites were predicted by using virtual atomic probes (shown as red spheres).

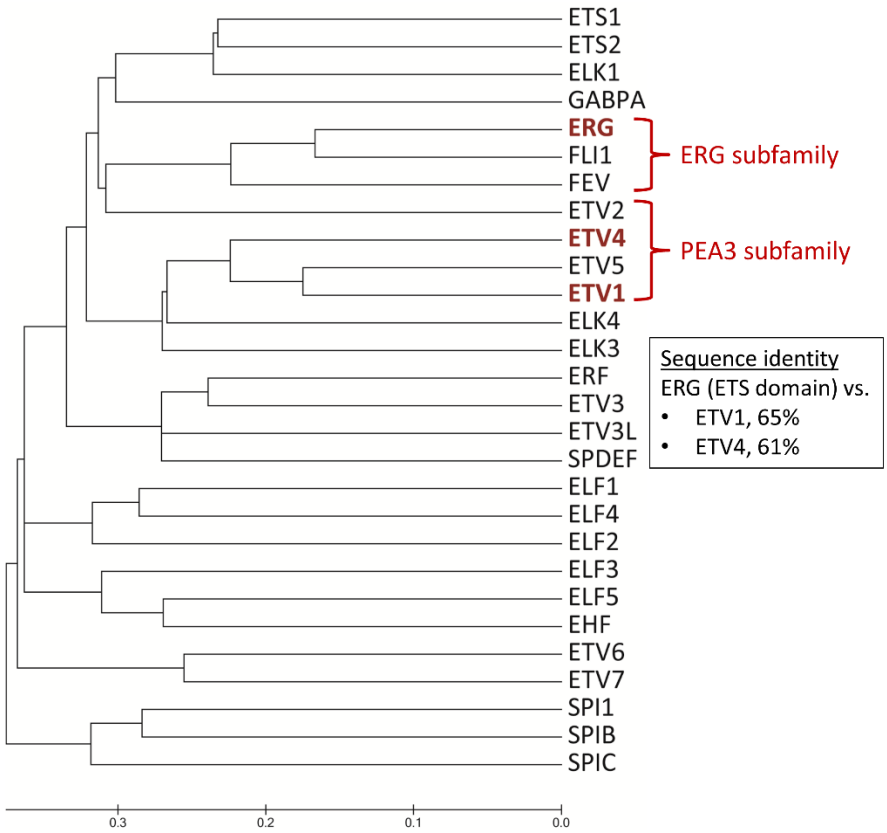


Figure 5. ETS phylogenetic tree. A phylogenetic tree of the 28 members in the ETS family, based on a full-length multiple protein sequence alignment.

Combining all the analysis results from the three major goals (genomic alteration, gene overexpression, protein structure availability and druggability, as well as supporting evidence accumulated in the literature[12,13,22-25] (**Table 3**), the three ETS factors (ERG, ETV1 and ETV4) have been identified as the top drug target candidates to be moved forward into Year 2 of the project, for the "Specific Aim 2 - Cheminformatics: development of new classes of anti-prostate cancer drugs".

Table 3. A summary of the three drug target candidates identified.

Drug target	Genomic alteration	Gene overexpression	Protein structure availability	Druggability	Supporting evidence in literature
ERG	Gene fusion	Fold-change ≥ 2	yes	yes	yes
ETV1	Gene fusion	Fold-change ≥ 1.3	yes	yes	yes
ETV4	Gene fusion	Fold-change ≥ 1.3	yes	yes	yes

4) Other achievements

The bioinformatic analyses on the nuclear receptors, in particular the orphan nuclear receptors, as potential drug targets in prostate cancer have resulted in a review paper[26] (attached in the Appendices).

Major Goal 4: Construct protein structural models and identify potential drug binding sites for each of three targets.

1) Major activities

As completed in Year 1 of the project for the *"**Specific Aim 1 - Genomics: identification of prostate cancer-related aberrations in gene regulation**"*, the three ETS factors (ERG, ETV1 and ETV4) have been identified as the drug targets based on the analysis results from the previous three major goals (genomic alteration, gene overexpression, protein structure availability and druggability, as well as supporting evidence accumulated in the literature). These three drug targets are transcription factors of the ETS protein family, known to be involved in cancer cell migration, invasion and metastasis[27,28].

To identify suitable protein structural templates, the protein sequence for each of the three drug targets has been searched against all the published structures in the Protein Data Bank[17] by using BLASTp[18]. The following X-ray crystal structures for the DNA-binding ETS domains have been identified for the three targets: ERG (PDB ID: 4IRG[29]), ETV1 (PDB ID: 4AVP[30]), and ETV4 (PDB ID: 4CO8[30]). Each of the published ETS domain X-ray crystal structures was subjected to the Site Finder algorithm, implemented in the Molecular Operating Environment (MOE), which used virtual atomic probes to search the protein surface for suitable small-molecule binding sites (or referred as pockets). The crystal structure of an ETS/DNA complex (PDB ID: 4IRI[29]) was used to determine the exact DNA interface. The top-ranked binding site was identified for each of the drug targets.

2) Specific objectives

The main objective of Major Goal 4 is to construct protein structure models for the identification of drug binding sites on the surface of each of the three drug targets (ERG, ETV1 and ETV4).

3) Significant results/key outcomes

As shown in **Figure 6**, the identified small-molecule binding pocket is located on the protein-DNA interface on each of the drug targets. It is anticipated that such small molecule binding will have the potential to sterically block DNA interaction with ERG, ETV1 and ETV4, disrupt their transcriptional activities, and inhibit downstream PCa transformational processes.

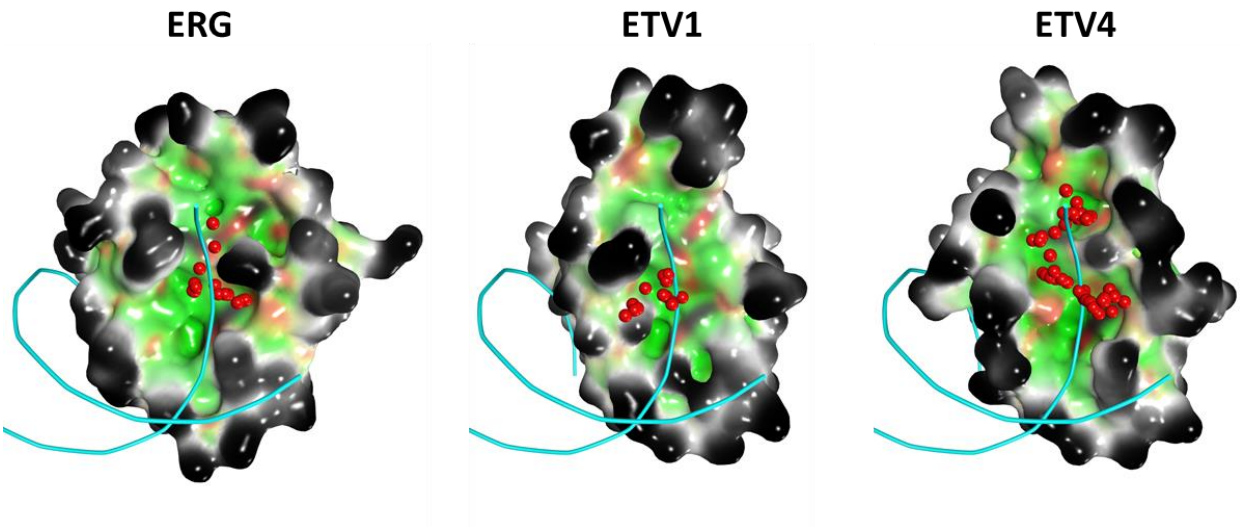


Figure 6. Drug binding pockets on the DNA-binding domain of ERG, ETV1 and ETV4. Protein surface models for the three drug targets: ERG, ETV1 and ETV4 are shown (colored according to exposed regions in black, hydrophobic regions in green, and polar regions in red). Drug (small-molecule) binding sites were determined by using virtual atomic probes (shown as red spheres). DNA backbones are shown as cyan ribbons.

Major Goal 5: Virtual compound screening against the binding pocket of drug target #1 (ERG).

1) Major activities

The top-ranked pocket identified for the ERG protein (**Figure 6**) was used for the virtual screening. Before molecular docking, the ERG-ETS domain structural model was prepared by using the Protein Preparation Wizard module of the Maestro v9.3 program from the Schrodinger 2012 software suite. The docking grid was centered at the pocket composed of the nearby amino acids. A virtual compound screening pipeline, based on protocols as previously established[31,32], has been utilized (**Figure 7**). A total of 20 million small molecule structures were downloaded from the ZINC database version 12[33]. Among the 20 million set, a total of 2 million molecules that possess the following lead-like and drug-like properties were extracted for molecular docking: molecular weight between 250 and 350 Da, logP <= 5, hydrogen-bond donors <= 5, hydrogen-bond acceptors <= 10, number of rotatable bonds <= 10, and number of rings <= 4. Each molecule was given its expected protonation state at pH 7 and energy-minimized under the MMFF94x (solvation: Born) force field using MOE. All of the 2 million molecules were compiled into a single SDF file as the input ligand database for the subsequent molecular docking step. Each molecule was docked into the previously defined docking grid on the ERG-ETS domain protein model, using the Glide program (Small-Molecule Drug Discovery Suite, version 5.8, Schrödinger 2012). Standard Precision with all other parameters set to default. The top 1% (~20,000 molecules), as ranked by the docking scores calculated based on interaction forces including hydrogen bonds and hydrophobic interactions, were selected to advance into the next stage of virtual screening. Within this set, a predicted pKi was calculated for each molecule using a custom MOE SVL script, and ligand efficiency was calculated using Glide. In addition, this set of 20,000 molecules was re-docked into the same pocket, using the eHiTs docking program[34]. A root-mean-square deviation (RMSD) was calculated between the docking poses from Glide and eHiTs for each molecule. A

consensus scoring (voting) method was used; each compound received one vote from each of the following criteria met: 1) top 20% pKi values, 2) top 20% ligand efficiency values, and 3) top 20% eHiTs docking scores and 4) RMSD <=3 A. The top 2,000 molecules, as ranked by the number of votes, were selected for the final stage of selection. During this step, the chemical structure of each molecule within the predicted ERG-ETS binding pocket was manually examined using the 3D visual environment in MOE. Preference was given to compounds with favorable binding poses and interactions with the surrounding amino acid residues. Molecules were removed from the selection if they contain problematic or promiscuous moieties. In addition to manual examination, the FAFDrugs program[35] was used to assist the identification of problematic chemicals.

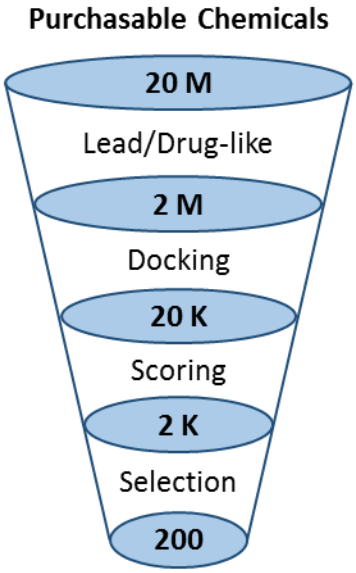


Figure 7. Virtual compound screening pipeline. M = millions, K = thousands.

2) Specific objectives

The main objective of Major Goal 5 is to perform virtual compound screening against the ERG pocket and to identify a list of candidate small molecules with high binding potential.

3) Significant results/key outcomes

By using the virtual screening pipeline as described (**Figure 7**), a total of 200 compounds with high binding potential against the drug target ERG were selected for further biological validation. As illustrated in **Figure 8**, a total of 2 million small molecules have been docked into the ERG pocket, with their corresponding docking scores calculated. Lower score represents higher binding potential. The scores ranged from -10.43 to 5.11, with a median value of -5.13. The 200 compounds selected all have their docking scores in the top 0.1%.

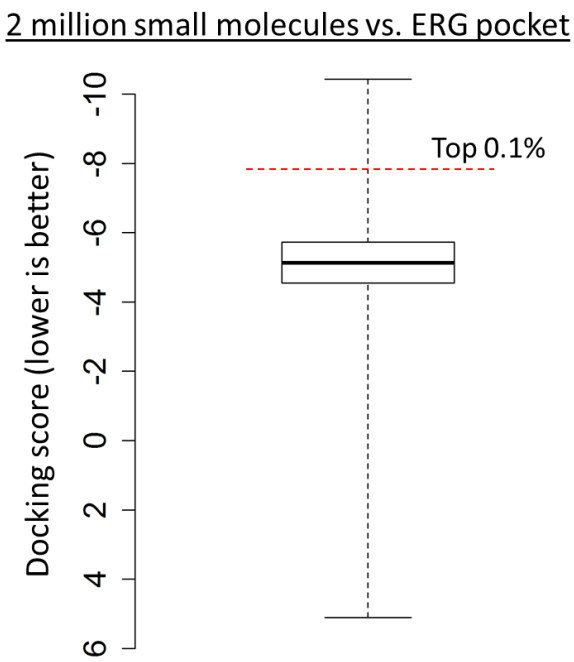


Figure 8. Docking score distribution of 2 million compounds vs. the ERG pocket. The 200 compounds selected for biological validation all have their docking scores in the top 0.1%.

Major Goal 6: Virtual compound screening against the binding pocket of drug target #2 (ETV1).

1) Major activities

The top-ranked pocket identified for the ETV1 protein (**Figure 6**) was used for the virtual screening, by following the same protocol as described in the previous method section of Major Goal 5 and in **Figure 7**.

2) Specific objectives

The main objective of Major Goal 6 is to perform virtual compound screening against the ETV1 pocket and to identify a list of candidate small molecules with high binding potential.

3) Significant results/key outcomes

By using the virtual screening pipeline as described (**Figure 7**), a total of 200 compounds with high binding potential against the drug target ETV1 were selected for further biological validation. As illustrated in **Figure 9**, a total of 2 million small molecules have been docked into the ETV1 pocket, with their corresponding docking scores calculated. Lower score represents higher binding potential. The scores ranged from -7.86 to 3.84, with a median value of -4.11. The 200 compounds selected all have their docking scores in the top 0.1%.

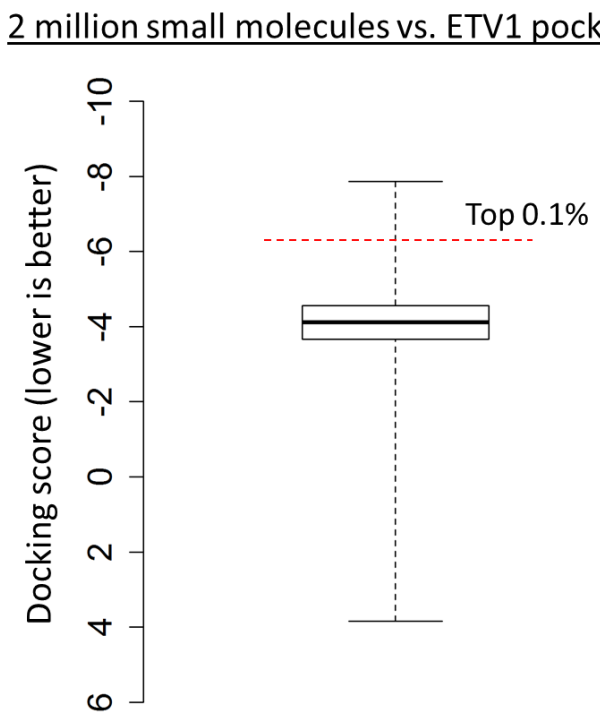


Figure 9. Docking score distribution of 2 million compounds vs. the ETV1 pocket. The 200 compounds selected for biological validation all have their docking scores in the top 0.1%.

Major Goal 7: Virtual compound screening against the binding pocket of drug target #3 (ETV4).

1) Major activities

The top-ranked pocket identified for the ETV4 protein (**Figure 6**) was used for the virtual screening, by following the same protocol as described in the previous method section of Major Goal 5 and in **Figure 7**.

2) Specific objectives

The main objective of Major Goal 7 is to perform virtual compound screening against the ETV4 pocket and to identify a list of candidate small molecules with high binding potential.

3) Significant results/key outcomes

By using the virtual screening pipeline as described (**Figure 7**), a total of 200 compounds with high binding potential against the drug target ETV4 were selected for further biological validation. As illustrated in **Figure 10**, a total of 2 million small molecules have been docked into the ETV4 pocket, with their corresponding docking scores calculated. Lower score represents higher binding potential. The scores ranged from -11.28 to 5.25, with a median value of -5.34. The 200 compounds selected all have their docking scores in the top 0.1%.

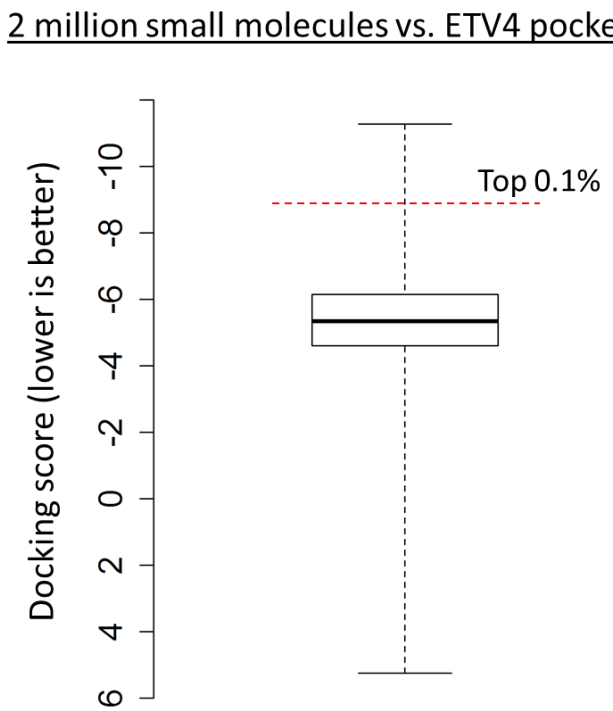


Figure 10. Docking score distribution of 2 million compounds vs. the ETV4 pocket. The 200 compounds selected for biological validation all have their docking scores in the top 0.1%.

Major Goal 8: Compound validations by biological assays.

1) Major activities

In collaboration with other research scientists at the Vancouver Prostate Centre, the 200 compounds identified from the virtual screening against each of the three drug targets (ERG, ETV1 and ETV4) were tested in luciferase-based ETS-responsive reporter assays, using three different cell lines: 1) VCaP that overexpresses ERG, 2) LNCaP that overexpresses ETV1, and 3) PC3 that overexpresses ETV4. Cells in 150 µL per well of a 96 well plate were seeded and after a 24 h incubation were transfected with 50 ng of ETS-responsive luciferase reporter[36] (Signosis) and 5 ng of the Renilla reporter (pRL-tk, Promega) using TransIT 20/20 transfection reagent (Mirus, USA). After 16 h incubation, cells were treated with compounds for a further 48 h. Luciferase and Renilla activities were measured using a TECAN M200Pro plate reader. Comparison of empty vector versus ETS-responsive reporter demonstrates activation only in the presence of the ETS responsive sequence. Data were normalized first to Renilla and then to the DMSO-media control on each plate. Initial hit compounds were identified as those with an average normalized luciferase reading (luciferase reading/Renilla reading) that is 60 % or less of the average normalized luciferase reading of the DMSO-media control (i.e. 40 % or more reduction of luciferase activity) at 25 µM.

2) Specific objectives

The main objective of Major Goal 8 is to identify a number of preliminary hit compounds with biological activities against the three drug targets: ERG, ETV1, ETV4.

3) Significant results/key outcomes

By testing those candidate compounds as previously identified from the virtual screening, some preliminary hit compounds have been found to inhibit luciferase reporter activities in PCa cells that overexpress ERG, ETV1 or ETV4 (**Figures 11-13)**. A published ERG inhibitor, YK-4-279 ('YK')[37], has been included in the assays as a comparison.

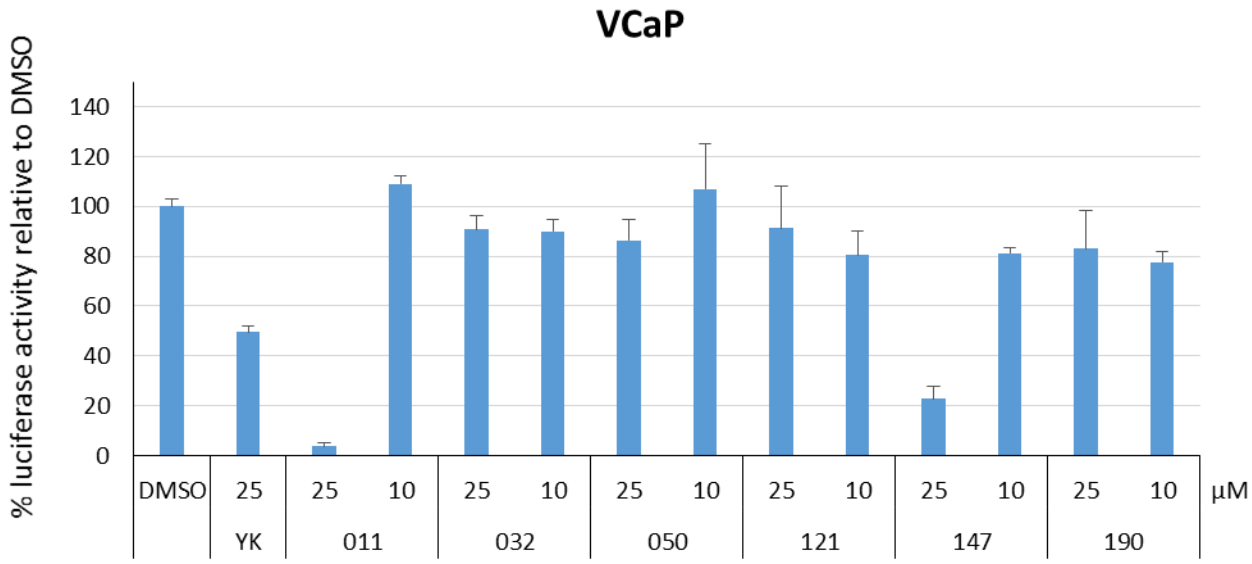


Figure 11. Representative compounds from the luciferase screening assay in VCaP cells. The assay has identified some preliminary hit compounds that inhibited the luciferase activity at 10 or 25 µM in VCaP cells that overexpressed ERG.

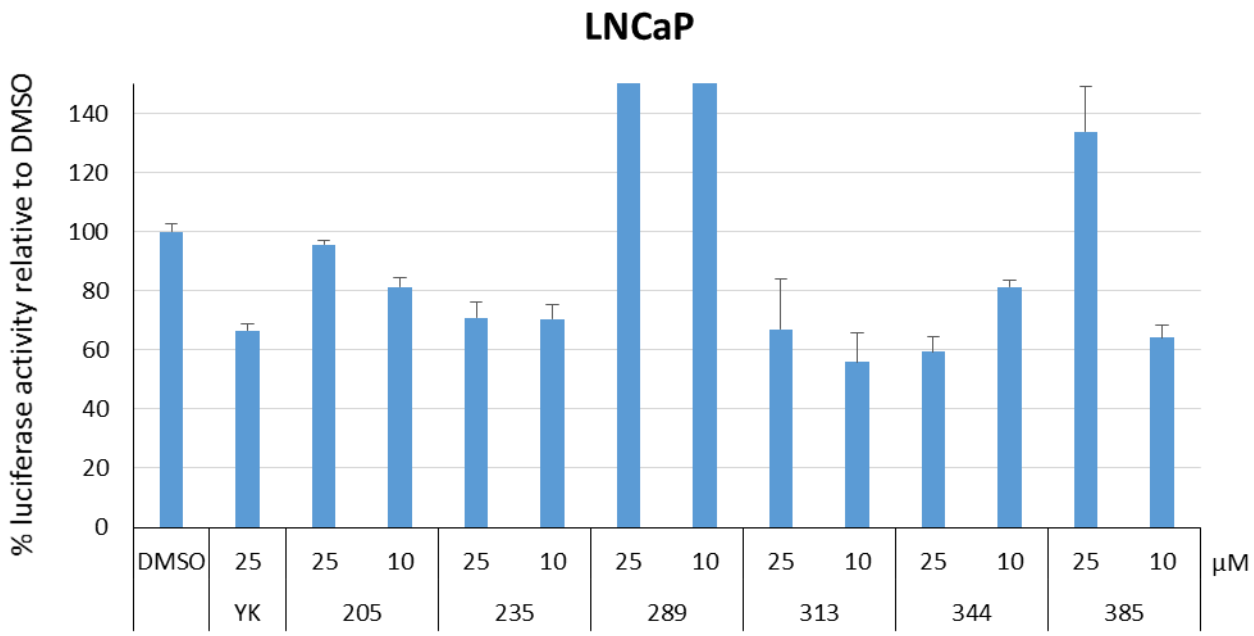


Figure 12. Representative compounds from the luciferase screening assay in LNCaP cells. The assay has identified a list of preliminary hit compounds that inhibited the luciferase activity at 10 or 25 μM in LNCaP cells that overexpressed ETV1.

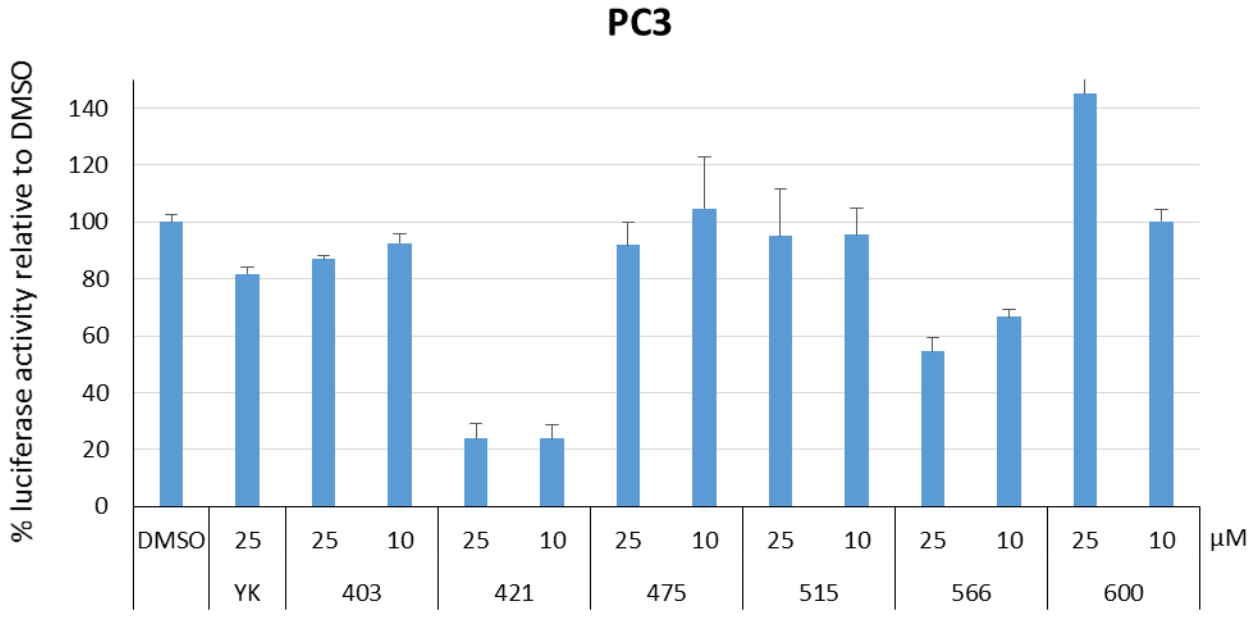


Figure 13. Representative compounds from the luciferase screening assay in PC3 cells. The assay has identified a list of preliminary hit compounds that inhibited the luciferase activity at 10 or 25 μM in PC3 cells that overexpressed ETV4.

In summary, experimental testing of these candidate compounds in the luciferase assays has identified several initial hits that inhibited the drug targets at 25 μM. Future work may include 1) repeating these preliminary hit compounds under multiple concentrations to establish a dose-dependent response and an IC_{50} value; 2) testing these hit compounds in cell viability assays to determine if the observed activity in the above luciferase experiments is due to any generic toxicity; and 3) testing non-toxic hits through additional *in vitro* assays such as cell migration and invasion.

What opportunities for training and professional development has the project provided?

Training activities:

This project has provided me (Dr. Michael Hsing, postdoctoral fellow) a great amount of opportunities for training and professional development. In order to enhance my knowledge in the field of cancer research (and in particular, prostate cancer), I have received personalized training from my mentor, Dr. Paul Rennie, who has over 30 years of research experience in prostate cancer and more than 180 peer-reviewed publications principally in this field. The training involved literature review of previous and current research on various topics in prostate cancer and other cancers as guided by Dr. Rennie. Cancer-related journal articles and textbooks were recommended by Dr. Rennie for studying. My knowledge in the field of prostate cancer and other cancers in general have been examined during weekly in-person meetings and email communication with Dr. Rennie. Furthermore, I have assisted Dr. Rennie for preparing and submitting a number of research grant applications.

Professional development activities:

Vancouver Prostate Centre (VPC), UBC is located at the largest research hub in Vancouver, adjacent to the Vancouver General Hospital, BC Cancer Agency (BCCA), and the Genome Science Centre. This research environment greatly facilitates research communication and collaboration among not only local scientists but also international researchers through regular seminars. I have learned about the latest cancer research findings and interacted with international cancer research scientists through a number of seminar series including weekly Terry Fox seminars hosted at VPC, bi-weekly seminars at BCCA, and monthly Pacific Northwest Prostate Cancer SPORE video conferences hosted by the Fred Hutchinson Cancer Research Center at Seattle, Washington. I have presented my research findings at regular group meetings and attended bi-weekly journal clubs at VPC.

During the course of the two year period, I have presented the research project at 1) Prostate Centre/ Terry Fox Seminar Series at Vancouver BC in February 2015; 2) American Association for Cancer Research (AACR) Annual Meeting at Philadelphia PA in April 2015, 3) Terry Fox Research Institute (TFRI)-BC Node Research Day at Vancouver BC in November 2015; 4) Vancouver Prostate Centre Science Social at Vancouver BC in February 2016; and 5) Annual Lorne D. Sullivan Lectureship & Research Day at Vancouver BC in June 2016. The genomic/bioinformatic part of the research project has resulted in the publication of a review article[26] (attached in the Appendices), with two additional manuscripts currently under preparation for the cheminformatic results.

How were the results disseminated to communities of interest?
Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
Nothing to Report.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Prostate cancer (PCa) is one of the most common cancers for males in the United States and Canada, with 245,000 cases and 32,000 deaths in 2015[1,2]. Major issues facing PCa patients are cancer recurrence due to the presence of distant metastasis at time of surgery and the development of treatment resistance. The proposed project is aimed to address this urgent issue of metastasis and drug-resistance in prostate cancer by using the combined power of genomic analyses, protein structural modeling, rational drug design and biological screening to develop new classes of anti-prostate cancer drugs, targeting previously under-appreciated proteins that play important roles in cancer progression.

To address a Prostate Cancer Research Program (PCRP) overarching challenge, "Develop effective treatments and address mechanisms of resistance for men with high risk or metastatic prostate cancer" with a focus area, "Therapy: Identification of new targets, pathways, and therapeutic modalities", the project has identified three drug targets (ERG, ETV1 and ETV4) based on large-scale and comprehensive analyses of available genomic and transcriptomic data in prostate tumor samples (as a result of the previous reporting period, Year 1). During this final reporting period (Year 2), each of the drug targets has been screened rigorously against millions of virtual chemical structures to identify candidate molecules with the best structural fit in the binding sites. The activity of these selected compounds have been validated by luciferase assays in collaborations with research scientists at Vancouver Prostate Centre (VPC), University of British Columbia. As a result, a list of preliminary hit compounds have been found to inhibit transcriptional activities driven by the three drug targets (ERG, ETV1 and ETV4). Future works are needed to further investigate these hit compounds using *in vitro* and *in vivo* PCa models.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change
Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report.

Changes that had a significant impact on expenditures
Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report.

Significant changes in use or care of human subjects
Nothing to Report.

Significant changes in use or care of vertebrate animals.
Nothing to Report.

Significant changes in use of biohazards and/or select agents
Nothing to Report.

6. PRODUCTS

Publications, conference papers, and presentations

Journal publications.
Roshan-Moniri M, **Hsing M**, Butler MS, Cherkasov A, Rennie PS. Orphan nuclear receptors as drug targets for the treatment of prostate and breast cancers. Cancer Treat Rev. (2014). 40: 1137-52. (Published).

Books or other non-periodical, one-time publications.
Nothing to Report.

Other publications, conference papers, and presentations.
Oral presentations:
Hsing M. Development of small molecule inhibitors of ERG to treat advanced prostate cancer. Prostate Centre/ Terry Fox Seminar Series. Jim Pattison Pavilion, Vancouver General Hospital, Vancouver, Canada. February 13, 2015. (Local). Acknowledgement of federal support (yes).

Poster presentations:
Roshan-Moniri M, **Hsing M**, Butler MS, Lau D, Axerio-Cilies P, Yen P, Kim A, Lien S, Mroczek M, Ma D, Li H, Guo Y, Ban F, Ghaidi F, LeBlanc E, McIntosh L, Cox ME, Cherkasov A, and Rennie P. Therapeutic targeting of ETS factor ERG for the treatment of prostate cancer. AACR Annual Meeting 2015. Pennsylvania Convention Center, Philadelphia, USA. April 18-22, 2015. (International). Acknowledgement of federal support (yes).

Butler MS, **Hsing M**, Roshan-Moniri M, Lau D, Yen P, Kim A, Lien S, Mroczek M, Ghaidi F, LeBlanc E, McIntosh L, Cox ME, Cherkasov A, and Rennie PS. Targeting ETS factor ETV4 as a novel therapeutic for the management of breast and prostate cancer. AACR Annual Meeting 2015. Pennsylvania Convention Center, Philadelphia, USA. April 18-22, 2015. (International). Acknowledgement of federal support (yes).

Butler MS, Roshan-Moniri M, **Hsing M**, Lau D, Axerio-Cilies P, Yen P, Kim A, Lien S, Mroczek M, Ma D, Li H, Guo Y, Ban F, Ghaidi F, LeBlanc E, Gregory-Evans C, McIntosh L, Cox M, Cherkasov A, and Rennie P. Therapeutic targeting of ETS factor ERG for the treatment of prostate cancer. The Terry Fox Research Institute (TFRI)-BC Node Research Day. Holiday Inn Vancouver Centre, Vancouver BC, Canada. November 16, 2015. (National). Acknowledgement of federal support (yes).

Butler MS, Roshan-Moniri M, **Hsing M**, Lau D, Kim A, Yen P, Mroczek M, Ghaidi F, Nouri M, Lawn S, Yamazaki T, Axerio-Cilies P, Gregory-Evans C, McIntosh L, Cox M, Rennie PS, and Cherkasov A. Therapeutic targeting of ETS factor ERG for the treatment of prostate cancer. Vancouver Prostate Centre Science Social. Vancouver BC, Canada. February 12, 2016. (Local). Acknowledgement of federal support (yes).

Butler MS, Roshan-Moniri M, **Hsing M**, Lau D, Kim A, Yen P, Mroczek M, Nouri M, Lien S, Yau C, Axerio-Cilies P, Ghaidi F, Dalal K, Guo Y, Lawn S, Yamazaki T, Fazli L, Gleave ME, Gregory-Evans CY, McIntosh LP, Cox ME, Rennie PS, and Cherkasov A. Discovery and characterization of small molecules targeting the ETS factor ERG in prostate cancer. 10th Annual Lorne D. Sullivan Lectureship & Research Day. Paetzold Health Education Centre. Vancouver General Hospital. Vancouver, BC. Canada. June 21, 2016. (Regional). Acknowledgement of federal support (yes).

Website(s) or other Internet site(s)
Nothing to Report.

Technologies or techniques
Nothing to Report.

Inventions, patent applications, and/or licenses
Nothing to Report.

Other Products
A central shared database, consisted of all the genomic and transcriptomic datasets collected during this reporting period, has been constructed and is accessible by other research scientists at the Vancouver Prostate Centre, University of British Columbia.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Michael Hsing
Project Role:	PI, Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	24
Contribution to Project:	Dr. Hsing has performed work in all the areas, as described in the approved SOW.
Funding Support:	

Name:	Paul Rennie
Project Role:	Mentor, Professor, Director
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	Dr. Rennie has provided the mentorship and guidance to Dr. Hsing over the course of the project.
Funding Support:	Canadian Cancer Society Research Institute, Canadian Institute of Health Research, Terry Fox Foundation.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report.

What other organizations were involved as partners?
Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS
None required (as indicated in the Award document).

9. APPENDICES

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Cancer Treatment Reviews 40 (2014) 1137–1152

Contents lists available at ScienceDirect

Cancer Treatment Reviews

journal homepage: www.elsevierhealth.com/journals/ctrv

Anti-Tumour Treatment

Orphan nuclear receptors as drug targets for the treatment of prostate and breast cancers

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ARTICLE INFO

Article history:
Received 20 March 2014
Received in revised form 10 October 2014
Accepted 13 October 2014

Keywords:
Orphan nuclear receptor
Nuclear receptor
Prostate cancer
Breast cancer
Cancer therapeutics
Androgen receptor
Estrogen receptor

ABSTRACT

Nuclear receptors (NRs), a family of 48 transcriptional factors, have been studied intensively for their roles in cancer development and progression. The presence of distinctive ligand binding sites capable of interacting with small molecules has made NRs attractive targets for developing cancer therapeutics. In particular, a number of drugs have been developed over the years to target human androgen- and estrogen receptors for the treatment of prostate cancer and breast cancer. In contrast, orphan nuclear receptors (ONRs), which in many cases lack known biological functions or ligands, are still largely under investigated. This review is a summary on ONRs that have been implicated in prostate and breast cancers, specifically retinoic acid-receptor-related orphan receptors (RORs), liver X receptors (LXRs), chicken ovalbumin upstream promoter transcription factors (COUP-TFs), estrogen related receptors (ERRs), nerve growth factor 1B-like receptors, and "dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1" (DAX1). Discovery and development of small molecules that can bind at various functional sites on these ONRs will help determine their biological functions. In addition, these molecules have the potential to act as prototypes for future drug development. Ultimately, the therapeutic value of targeting the ONRs may go well beyond prostate and breast cancers.

Introduction

The nuclear receptor (NR) superfamily is composed of transcriptional factors that enable multicellular organisms to regulate gene expression in response to a wide variety of stimuli from developmental, physiological and environmental sources. NR actions are modulated by several endogenous mechanisms that include: (1) ligand binding, (2) posttranslational modification of amino acid residues, (3) protein dimerization, (4) nuclear transfer, (5) protein–protein interactions with activators and repressors, and (6) cooperative DNA binding with other transcription factors [1]. Of the 48 members of the superfamily, 36 receptors were originally described as orphan NRs (ONRs) because their exact function and native ligands were undefined [2]. As a result of recent research efforts, 21 of these ONRs became annotated as "adopted" due to the discovery of natural or synthetic ligands (Fig. 1A).

NRs are commonly classified into 7 subfamilies based on their sequence similarity [3] and have been studied for their associations with cancers in a wide range of human organs (Table 1). However, there is a disparity of research efforts dedicated to ONRs, as illustrated by the number of published articles in PubMed (Fig. 1B). For example, during the year 2013 alone, 543 and 1312 articles were published on the androgen receptor (AR) and estrogen receptor (ER) in the context of their corresponding cancers. By comparison, less than 30 articles were published in the same year on the role of each ONR in cancer, as highlighted by Fig. 1C. This imbalance in research activity is also mirrored in the number of drugs and chemicals targeting the ONRs (Fig. 1A).

Conventionally, targeting transcriptional factors has been a challenging task in drug discovery due to the lack of well-defined active sites [4]. Thus, the unique presence of the ligand binding domain in NRs has made them very attractive drug targets, and NRs are among the top four families of drug targets and account for approximately 13% of all Food and Drug Administration (FDA)-approved drugs [5]. To gain insight into the number of existing small molecules targeting the NR family, each NR was queried based on its gene symbol, protein name and protein sequence

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http://dx.doi.org/10.1016/j.ctrv.2014.10.005
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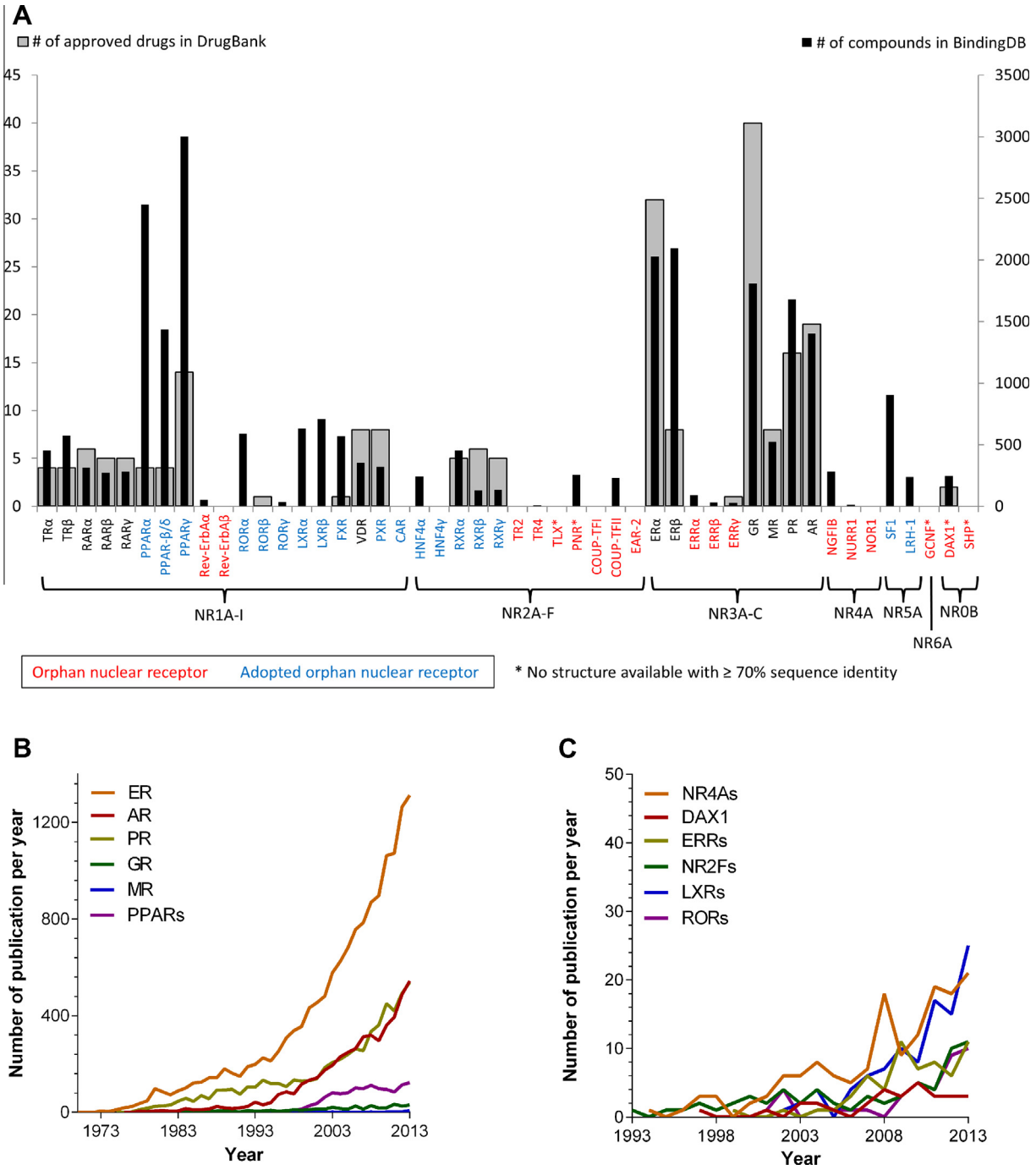


Fig. 1. Comparison of existing approved drugs, experimental compounds and publications for NR and ONRs. (A) Each NR was queried for the number of approved drugs (the left *y*-axis shown in gray bar) in DrugBank [6], and for the number of published compounds (the right *y*-axis shown in black bar) with experimentally-determined binding affinities in BindingDB [7] in October 2013. The NRs are arranged according to the subfamily as appeared in Table 1, and further classified as orphan or adopted ONR according to Shi et al. [2] Their protein sequences were compared with those with existing experimental structures in PDB [25] by using standard protein BLAST [40]. NRs that have no structural template with sequence identity higher than 70% are marked by '*'. The number of articles published on (B) the five hormone receptors (ER, AR, PR, GR and MR) and PPARs and (C) the ONRs discussed in this review paper, were extracted from PubMed in December 2013 using a search query consisted of: (Cancer [Title/Abstract]) AND ("Name of the nuclear receptor"[Title/Abstract]). Gene names and synonyms were used wherever applicable.

through the DrugBank [6] and BindingDB [7] databases. The DrugBank contains information on existing drugs and their direct targets, including 1541 FDA-approved small molecule therapeutics and 5082 experimental drugs that interact with 4323 proteins. BindingDB currently contains over one million binding affinity records corresponding to 427,325 small molecules and 6589 protein targets, extracted from the literature. As illustrated in Fig. 1, five hormone NRs (AR, ER, glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and progesterone receptor (PR)) are associated with a large number of synthetic inhibitors developed for the treatment of prostate- and breast cancers among other diseases [8,9]. Similarly, some adopted ONR such as peroxisome proliferator-activated receptors (PPARs) have received considerable attention and have clinically approved drugs for the treatment of diabetes and obesity [2]. In contrast, most other members of the ONR family have fewer associated inhibitors identified due to

Table 1
Nuclear receptors in cancers.

Subfamily	Group name	Abbreviation	Cancer association
1. Thyroid hormone receptor-like	Thyroid hormone receptor (NR1A)	TRα TRβ	Liver [166], Kidney [167], Pituitary [168], Breast [169], Thyroid [170]
	Retinoic acid receptor (NR1B)	RARα RARβ RARγ	Leukemia [171], Breast [172], Head &Neck [173], Lung [174], Oral tissue [175], Cervix [176], Ovary [177], Esophageal [178], Prostate [179]
	Peroxisome proliferator-activated receptor (NR1C)	PPARα PPAR-β/δ PPARγ	Brain [180], Lung [181], Stomach & Colon [182], Liver [183], Pancreas [184], Bladder [185], Breast [186], Testis [187], Bone [188]
	Rev-ErbA (NR1D)	Rev-ErbAα Rev-ErbAβ	N/A
	RAR-related orphan receptor (NR1F)	RORα RORβ RORγ	Lymphoma [189], Breast [73]. Prostate [69]
	Liver X receptor-like (NR1H)	LXRα LXRβ FXR	Prostate [87], Breast [190], Colon [191], Pancreas [192], Esophageal [193], Liver [194]
	Vitamin D receptor-like (NR1I)	VDR PXR CAR	Colon [195], Prostate [196], Breast [197] Colon [198], Breast [199], Ovary [200] Liver [201]
2. Retinoid X receptor-like	Hepatocyte nuclear factor (NR2A)	HNF4α HNF4γ	Liver [202], Colon [203], Breast [204], Prostate [205]
	Retinoid X receptor (NR2B)	RXRα RXRβ RXRγ	Breast [146] Colon [206]
	Testicular receptor (NR2C)	TR2 TR4	N/A
	TLX/PNR (NR2E)	TLX PNR	N/A
	COUP/EAR (NR2F)	COUP-TFI COUP-TFII EAR-2	Prostate [106], Lung [207] Breast [108]
3. Estrogen receptor-like	Estrogen receptor (NR3A)	ERα ERβ	Breast [9], Cervix [208], Colon [209], Liver [210], Lung [211], Pancreas [212], Prostate [213], Thyroid [214], Esophageal [215], Ovary [216], Adrenocortical [217]
	Estrogen related receptor (NR3B)	ERRα ERRβ ERRγ	Prostate [115], Breast [218]
	3-Ketosteroid receptors (NR3C)	GR MR PR AR	Prostate [219], Breast [220], Lung [221], Pancreas [222] N/A Ovary [223], Endometrial [224], Gallbladder [225], Breast [226] Prostate [8], Breast [227]
4. Nerve growth factor IB-like	NGFIB/NURR1/NOR1 (NR4A)	NGFIB NURR1 NOR1	Breast [228], Colon [229], Prostate [143]
5. Steroidogenic factor-like	SF1/LRH1 (NR5A)	SF1 LRH-1	Prostate [230], Adrenocortical Tumors [231] Breast [232], Colon [233]
6. Germ cell nuclear factor-like	GCNF (NR6A)	GCNF	N/A
0. Miscellaneous	DAX/SHP (NR0B)	DAX1 SHP	Bone [234], Breast [155], Prostate [121]

Nomenclature used is based on the system defined by the Nuclear Receptors Nomenclature Committee [235].

difficulties in drug development and toxicity, as well as, the continued focus on the identification of natural ligands [10]. For example, the role of retinoid X receptors (RXRs) as heterodimeric partners of several members of the nuclear receptor family, including retinoic acid receptor (RAR), makes them promising drug targets; however, controversy still surrounds whether or not 9-*cis*-retinoic acid is its true endogenous ligand [11].

To bring attention to ONRs as prospective novel cancer drug targets, this article reviews general structural features and biological functions of ONRs and discusses some of the most promising candidates for therapeutic targeting in both prostate and breast cancers.

Targeting different functional sites in NRs with small molecules

All NRs are modular in nature and composed of six conserved regions, designated A-F, which make up the following domains: (A/B) the N-terminal regulatory domain (NTD), (C) DNA binding domain (DBD), (D) hinge region, (E) ligand binding domain (LBD), and (F) C-terminal domain. A phylogenetic tree was constructed based on a multiple alignment of full-length protein sequences of all 48 NRs using the Clustal Omega [12] software, and it demonstrated that each NR can be further grouped into the corresponding subfamilies based on sequence similarities (Fig. 2). In addition, Fig. 2 features sequence identity between each NR and AR/ER at the DBD and LBD, and it highlights that DBDs are more conserved, sharing ~50% sequence identity, while the LBD is less conserved (~25% sequence identity). The extent of sequence conservation within the DBD and LBD regions are further depicted as a heat map in Fig. 3A.

Among the six domains, the NTD of nuclear receptors contains a transcriptional activation function-1 site (AF-1), and its activity does not directly depend on the presence of activating ligands [13,14]. Although the transcriptional activity of AF-1 is limited, it is known to synergize with the ligand-regulated activation

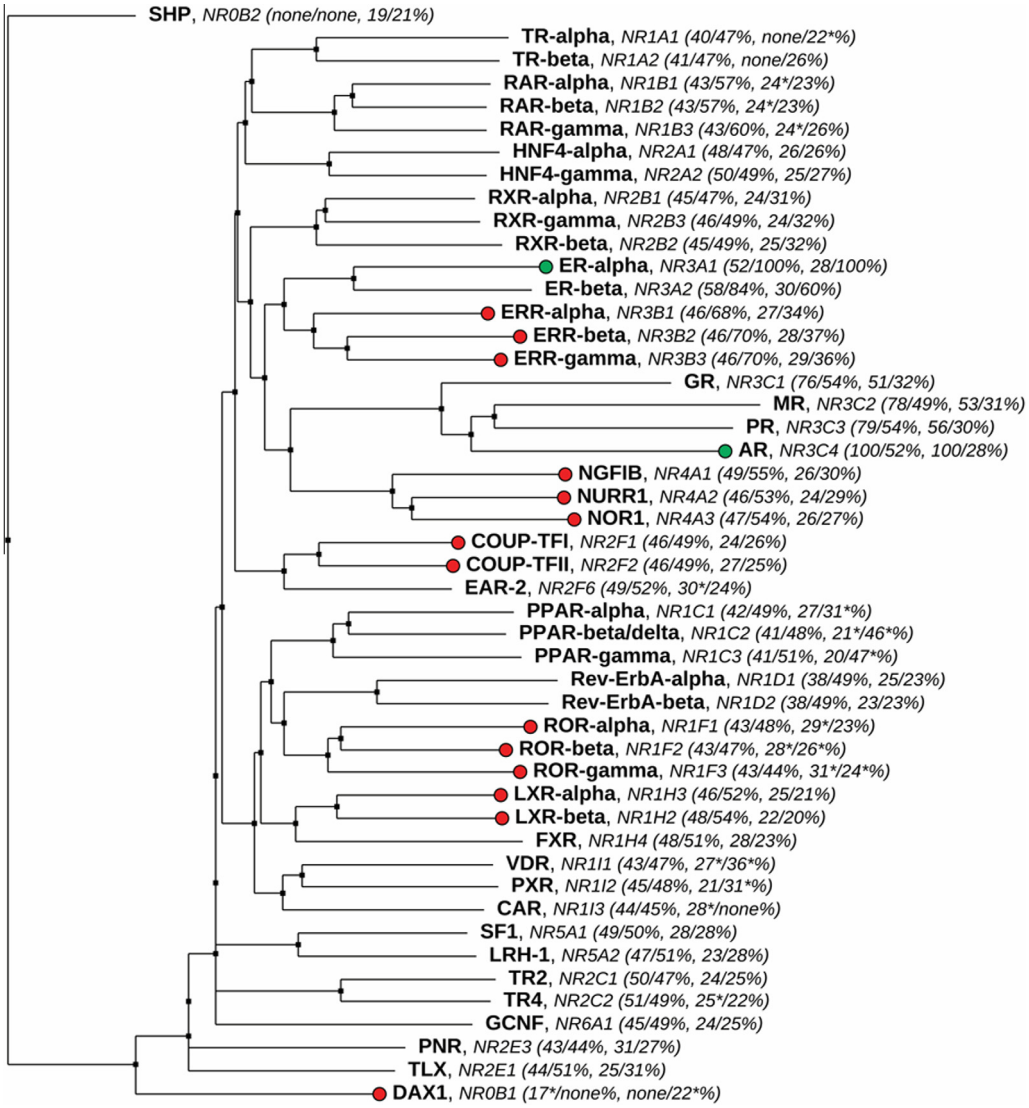


Fig. 2. A phylogenetic tree of NRs. A phylogenetic tree was constructed based on a multiple alignment of full-length protein sequences of all 48 NRs using Clustal Omega [12]. The ONRs featured in the review paper are highlighted in red, and the two nuclear receptors, AR and ERα, are indicated in green as the reference. The sequence identity of each NR is compared to AR and ERα at the DBD and LBD, with the domain boundary positions defined by NCBI Conserved Domain Database (CDD) [161]. The following format is used (a/b%, c/d%): a = % identity vs. AR-DBD, b = % identity vs ERα-DBD, c = % identity vs AR-LBD, and d = % identity vs ERα-LBD. * = query alignment coverage is less than 50%.

function-2 (AF-2) regulatory site located within the LBD to regulate gene expression. Significant variability in the NTD can be observed even within a single type of NR where different sequences can be derived from alternative splicing, promoters, and transcription start sites. One of the best examples of such variability is found with RAR where three gene templates, designated α, β and γ yield eight alternative protein variants [15,16].

The DBD has the highest sequence conservation among the NRs, and it mediates receptor binding to response elements (REs) located in the enhancers and promoters upstream of NR-regulated genes. The receptors can bind to REs as a monomer, homodimer or heterodimer, and the two zinc finger motifs within the DBD aid RE binding and receptor dimerization [17]. All NRs contain a DBD, with the exception of dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX1) and small heterodimer partner (SHP), both of which lack a DNA binding domain [18,19]. The absence of DBD on DAX1 and SHP can also be demonstrated by the lack of sequence alignment in Fig. 3A.

The hinge region is the connecting bridge between the DBD and LBD sections. The sequences of the hinge region vary significantly among NRs and contribute to flexible rotations of the DBD, enabling direct or inverted binding and dimerization of NRs onto the HREs [20]. There is some evidence that this region may also serve as a binding site for co-repressor proteins [21,22].

The LBD has only moderate sequence conservation among NRs, but possess highly similar three-dimensional organizations. The LBD is composed of 11–12 alpha helices, which can fold into a compact formation with parallel and anti-parallel orientation (Fig. 3B). This domain is associated with multiple functions including ligand attachment through the ligand-binding site (LBS), interactions with other proteins at the AF-2 and binding function-3 (BF-3) co-regulator binding sites, protein dimerization, and nuclear translocation [23]. For more in-depth discussions on AF-2 and BF-3 sites in NRs, we refer readers to the two recent review papers by Buzon et al. [24] and Caboni and Lloyd [17].

In additional to high-throughput screening and other conventional approaches, computer-aided drug design, guided by available protein structures in the Protein Data Bank (PDB) [25] and synthetic compounds from databases such as ZINC [26] can provide a cost- and time-effective strategy to search for new drug

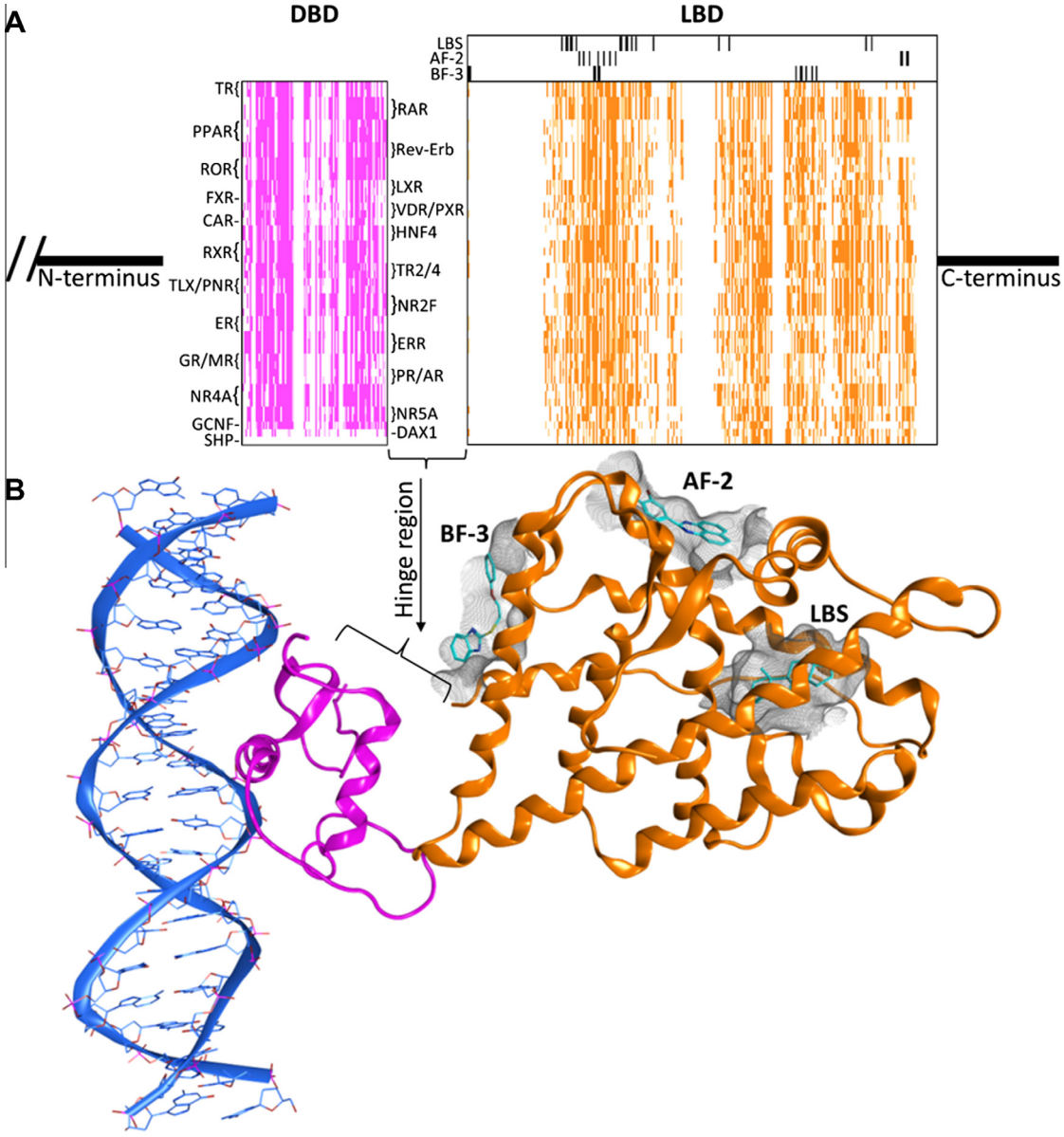


Fig. 3. Sequence conservation and a general 3D structural model of protein domains in nuclear receptors. (A) A heat map at the top panel illustrates a multiple protein sequence alignment among the 48 NRs at the DNA binding domain (DBD) and ligand binding domain (LBD), generated by using Clustal Omega [12] and visualized in Jalview [162]. The domain boundary positions are defined according to NCBI CDD [161]. A darker color represents a residue that matches the consensus sequence (more conserved) at a position, while a lighter color represents a different residue but with a positive Blosum62 score (less conserved). A non-conserved residue or a gap is colored in white. The 48 NRs are arranged from top to bottom (*y*-axis) in the same order as they appear in Table 1. The aligned residues are arranged from the N-terminus to the C-terminus on the *x*-axis. Residues within the LBS, AF-2 and BF-3 sites are highlighted as black bars on top of the LBD heat map, using the AR sequence as the reference positions defined by NCBI CDD [161] and Buzon et al. [24]. (B) The bottom panel shows a general 3D structural model, reconstructed by combining several crystal structures of individual domains, which include: (1) DBD (in pink ribbon) bound at DNA (PDB ID: 1R4I) [163]; (2) the relative orientation among DBD, the hinge region and LBD (PDB ID: 4IQR) [164]; (3) LBD (in orange ribbon) and the ligand testosterone bound at the ligand-binding site (PDB ID: 2AM9) [165]; (4) LBD with a small molecule inhibitor bound at the AF-2 site (PDB ID: 2YHD) [35]; and (5) LBD with a small molecule inhibitor bound at the BF-3 site (PDB ID: 4HLW) [37]. No structural model of the N-terminal regions is available. Protein models were visualized by using the Molecular Operating Environment (MOE).

candidates and has been successfully applied to a number of NRs [17,23]. For instance, virtual screening methods have been used to discover agonists and antagonists targeting the ligand binding domains of PPAR [27], RAR [28], estrogen related receptor (ERR) [29] and Liver receptor homolog 1 (LRH-1) [30]. While many existing drugs targeted the ligand binding site within the LBD of NRs, such as Enzalutamide [31] and Bicalutamide [32] for AR and Tamoxifen [33] and Fulvestrant [34] for ER, there are several new inhibitors that have been developed to target protein–protein interactions at alternative sites, as exemplified by AF-2 [35] and BF-3 [36,37] of the androgen receptor (Table 3). These alternative sites (depicted in Fig. 3B) are required for physical interactions with protein co-factors; thus, binding of small molecules at such sites can block co-factor interactions and provide additional means for inhibiting NRs, particularly in cases where drug resistance has occurred due to mutations in the LBS. Furthermore, it has been shown recently as a proof of concept that the protein-DNA interface on the DBD of AR can be targeted by small molecules [38,39], and this work opens up a new strategy to inhibit different protein variants that may differ in LBD but share the same DBD within the AR or in other NR subfamilies.

To evaluate the "druggability" and the number of available structural templates of each NR, protein sequences were aligned with those with existing protein structures in PDB [25] by using

Table 2
A list of orphan nuclear receptors featured in this review paper.

Name	UniProt ID	Endogenous ligands	Interactors (BioGRID)	Protein structures (PDB ID)	LBS/AF-2/BF-3 vs. AR (sequence identity, similarity)
RORα	P35398	Cholesterol, cholesterol sulfate, hydroxycholesterol, ketocholesterol, epoxycholesterol, ruscogenin [64]	24 Interactors (ID: 112022)	1N83, 1S0X	6/27/17%, 24/64/25%
RORβ	Q92753	Stearic acid, retinoic acid [64]	14 Interactors (ID: 112023)	1K4W*, 1NQ7*, 1N4H*	6/27/17%, 24/64/25%
RORγ	P51449	Hydroxycholesterol, ketocholesterol, epoxycholesterol [64]	3 Interactors (ID: 112024)	3B0W, 3KYT, 3L0J, 3L0L, 4NB6, 4NIE	12/27/25%, 29/64/33%
LXRα	Q13133	Oxysterols [90]	37 Interactors (ID: 115373)	1UHL, 3IPQ, 3IPS, 3IPU	18/36/25%, 35/64/25%
LXRβ	P55055	Oxysterols [90]	54 Interactors (ID: 113222)	1P8D, 1PQ6, 1PQ9, 1PQC, 1UPV, 1UPW, 3KFC, 3L0E, 4DK7, 4DK8	18/36/25%, 35/64/25%
COUP-TFI	P10589	Orphan	20 Interactors (ID: 112883)	2EBL	18/9/17%, 35/73/25%
COUP-TFII	P24468	Orphan	30 Interactors (ID: 112884)	3CJW	18/9/17%, 35/73/25%
ERRα	P11474	Orphan	44 Interactors (ID: 108405)	1XB7, 2PJL, 3D24, 3K6P	35/45/25%, 76/91/33%
ERRβ	O95718	Orphan	7 Interactors (ID: 108406)	1LO1	35/45/25%, 71/91/42%
ERRγ	P62508	Orphan	24 Interactors (ID: 108407)	1KV6, 1TFC, 1VJB, 2E2R, 2EWP, 2GP7, 2GPO, 2GPP, 2GPU, 2GPV, 2P7A, 2P7G, 2P7Z, 2ZAS, 2ZBS, 2ZKC	35/45/25%, 71/91/33%
NGFIB	P22736	Orphan	84 Interactors (ID: 109407)	2QW4, 3V3E, 3V3Q, 4JGV, 4KZI, 4KZJ, 4KZM	12/9/33%, 47/45/33%
NURR1	P43354	Orphan	14 Interactors (ID: 110983)	1OVL	18/0/33%, 47/45/42%
NOR1	Q92570	Orphan	4 Interactors (ID: 113713)	1CIT*	18/0/33%, 47/45/42%
DAX1	P51843	Orphan	15 Interactors (ID: 106695)	3F5C**	6/27/17%, 41/100/33%

The lack of endogenous ligands in ONRs was confirmed by querying the IUPHAR/BPS database [236] in October 2014. BioGRID: the number of interactors queried in October 2014. PDB: available protein structures queried in July 2014. Protein structure: *homologous protein structures with $\geqslant$ 70% sequence identity. **homologous protein structures with <70% sequence identity. Sequence similarity was calculated based on the Blosum62 matrix vs. AR sequences. Residue positions within each functional site, LBS (n = 17), AF-2 (n = 11) and BF-3 (n = 12), were defined based on the AR protein, according to NCBI Conserved Domain Database (CDD) [161] and Buzon et al. [24].

a standard BLAST algorithm [40]. As highlighted in Fig. 1 and Table 2, the majority of NRs and ONRs have protein structural templates with sequence identity more than 70%, which is sufficient for homology modeling as a basis for virtual screening. The multiple sequence alignment as represented as a heat map in Fig. 3A illustrates that the positions of amino acids at LBS, AF-2 and BF-3 sites overlap with highly conserved regions within LBD. This is in accordance with previous reports [24] indicating that these functional sites are often conserved among many NRs including members of the ONR family [41–45] and as such may be targetable. To facilitate future drug design targeting the ONRs, Table 2 shows a list of ONRs featured in this review paper and their annotations including accession numbers in the UniProt database [46], endogenous ligands, the number of interactors listed in the BioGRID database [47], and available protein structures in PDB. To determine the extent of sequence conservation at the LBS and possible presence of AF-2 and BF-3, the corresponding residues of each ONR from the multiple sequence alignment were compared to those of the androgen receptor, and the sequence identity/similarity values were listed in Table 2. Interestingly, the AF-2 site of RORs, LXRs, COUP-TFs, ERRs and DAX1 share moderate to high sequence similarities with the AR (64%, 64%, 73%, 91% and 100% respectively), and previous studies have confirmed the presence of AF-2 in RORs and ERRs [48,49].

In the next sections, we focus on these ONRs as potential drug targets in prostate and breast cancers. For each ONR, we review its functions in normal physiological and cancer-related conditions. The druggability of each highlighted ONR and known small molecule ligands are also discussed. Table 3 summarizes a selected list of existing drugs and experimental compounds targeting each ONR, while synthetic compounds targeting AR and ER are also included for reference. For the roles of other NRs and ONRs in different cancers, we refer readers to a list of representative articles summarized in Table 1.

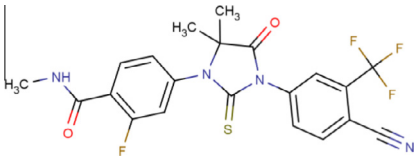
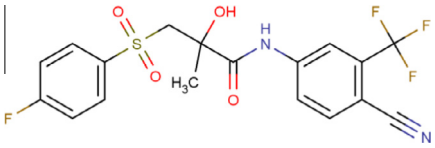
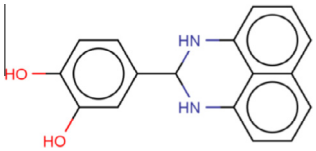
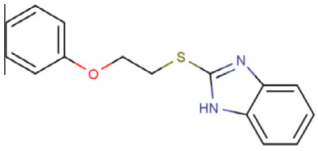
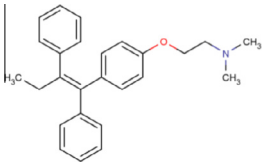
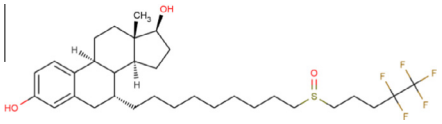
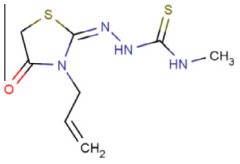
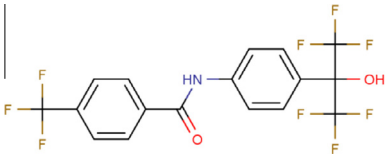
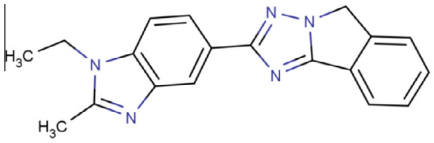
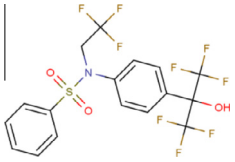
Targeting ONRs in prostate and breast cancers

Prostate cancer is one of the most common cancers in men, with an estimated 238,590 newly diagnosed cases and 29,720 deaths in 2013 in the United States alone [50]. As a result of improved screening procedures, prostate cancers are currently being diagnosed earlier, when the disease is localized to the prostate; if detected early, the treatment options of radical prostatectomy and/or radiation therapy are potentially curative. However, the main treatment options for advanced or metastatic prostate cancers are hormonal therapies.

Prostate cancer, like the non-malignant prostate, is dependent on androgens for proliferation and survival. Thus, hormonal therapies that aim to either reduce androgen levels (via surgical or chemical castration) or inhibit the AR protein (by small molecules) are used to slow tumor growth. Unfortunately, despite initial response, such treatments will eventually fail and the cancer recurs in an incurable, castration-resistant form [51,52]. This treatment resistance may be a result of several mechanisms including amplification of AR expression, mutations at the androgen-binding site of the AR and elevated production of AR splice variants [53]. Thus, the development of new therapeutics to combat metastatic castration-resistant prostate cancer remains a major task.

Breast cancer is the leading cause of cancer-related death in women worldwide [54]. In 2013 alone, there were an estimated

Table 3
A selected list of compounds targeting nuclear receptors.

Compound name	Chemical structure	Annotation (protein target/accession number/status/activity)
Enzalutamide		– AR: LBS antagonist [31] – DB08899 (DrugBank) – Approved, prostate cancer
Bicalutamide		– AR: LBS antagonist [32] – DB01128 (DrugBank) – Approved, prostate cancer
4-(2,3-Dihydro-1H-perimidin-2-yl)benzene-1,2-diol		– AR: AF-2 antagonist [35] – CID 761631 (PubChem) – Experimental, prostate cancer – IC_{50} = 18.64 μM (eGFP transcriptional assay)
2-[(2-Phenoxyethyl)sulfanyl]-1H-benzimidazole		– AR: BF-3 antagonist [37] – CID 1944110 (PubChem) – Experimental, prostate cancer – IC_{50} = 4.2 μM (eGFP transcriptional assay)
Tamoxifen		– ERs: [33] LBS, AF-2 antagonist – DB00675 (DrugBank) – Approved, breast cancer
Fulvestrant		– ERs: LBS antagonist [34] – DB00947 (DrugBank) – Approved, breast cancer
CGP 52608		– RORα agonist [69] – CID 6509863 (PubChem) – Experimental
SR1078		– RORα and RORγ agonist [71], [72] – CID 17980288 (PubChem) – Experimental
AC1M6FQE		– RORα antagonist (PubChem BioAssay AID: 610) – CID 2347892 (PubChem) – Experimental – IC_{50} < 5.03 nM (luciferase reporter assay)
T0901317		– LXRs: LBS agonist [93] – CID 447912 (PubChem) – Experimental – EC_{50} = 10 nM (fluorescence polarization assay), LXRα [237]

(*continued on next page*)

Table 3 (*continued*)

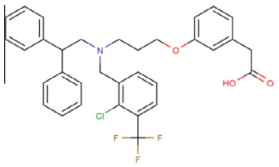
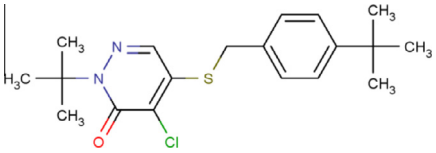
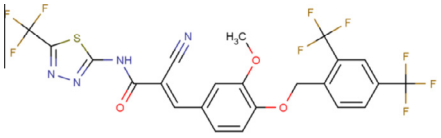
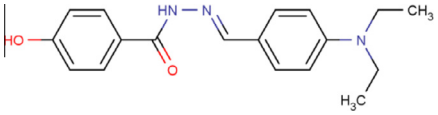
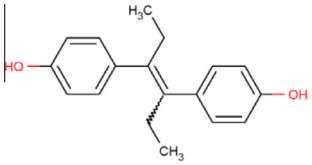
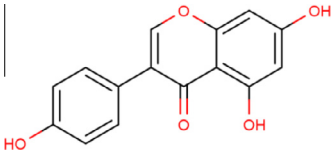
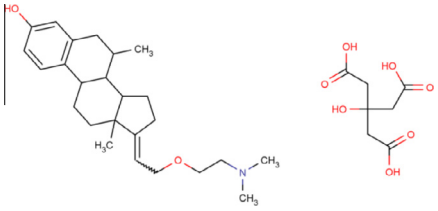
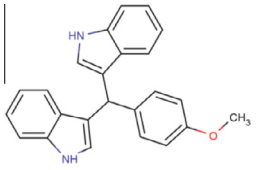
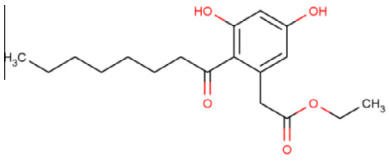
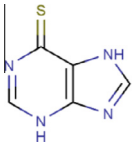
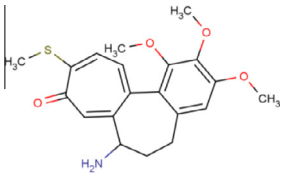
Compound name	Chemical structure	Annotation (protein target/accession number/status/activity)
GW3965		– LXRs agonist [88] – CID 447905 (PubChem) – Experimental – EC50 = 40 nM (FRET based cell-free ligand sensing assay), LXRβ (PubChem BioAssay AID: 437600)
Pyridaben		– COUP-TFII antagonist (PubChem BioAssay AID: 720548) – CID 91754 (PubChem) – Experimental – IC_{50} = 61.93 nM (luciferase reporter assay)
XCT790		– ERRα inverse agonist [113] – CID 6918788 (PubChem) – Experimental – IC_{50} = 0.37 μM (GAL4-ERRα cell-based transfection assay) [130]
DY131		– ERRβ/γ agonist [114,115] – CID 5497124 (PubChem) – Experimental – EC_{50} = 0.13 μM (FRET assay), ERRγ (PubChem BioAssay AID: 246440)
Diethylstilbestrol		– ERRγ antagonist [128] – DB00255 (DrugBank) – Approved, menopausal/postmenopausal disorders
Genistein		– ERRs agonist [29] – CID 5280961 (PubChem) – Natural compound
SR16388		– ERRα antagonist [131] – CID 54612678 (PubChem) – Experimental/Clinical trial
DIM-C-pPhOCH3 (C-DIM)		– NGFIB agonist/antagonist [133] – CID 11371604 (PubChem) – Experimental
Cytosporone B		– NGFIB agonist [142] – CID 10687292 (PubChem) – Natural compound – EC_{50} = 0.115 nM (luciferase reporter assay), PubChem, BioAssay AID: 500599
6-Mercaptopurine		– NGFIB-like agonist [148] – DB01033 (DrugBank) – Approved, antineoplastic agent

Table 3 (*continued*)

Compound name	Chemical structure	Annotation (protein target/accession number/status/activity)
Thiocolciran		– DAX1 antagonist (PubChem BioAssay AID: 687017) – CID 426728 (PubChem) – Experimental – IC_{50} = 212.03 nM (luciferase reporter assay)

Accession numbers are provided for either DrugBank [6] or PubChem [224], and chemical structures were visualized by using MarvinSketch.

232,340 newly diagnosed cases and 39,620 deaths in the United States [50]. Based on the gene expression profiles, breast cancers can be divided into five subtypes that vary in their treatment options and survival outcomes [55–58]. ERα positive (ERα+) and progesterone receptor positive (PR+) tumors account for approximately 70% of all cases [59,60]. Additionally, based on the expression or gene amplification of the ErbB-2/HER2 gene, these ERα+/PR+ tumors can be further classified into HER2− and HER2+ subtypes. For both early and advanced ERα+ breast cancer, the standard treatment is to block the production of estrogens (e.g. aromatase inhibitors) or estrogen binding to the receptor (e.g. Tamoxifen). Adjuvant Trastuzumab therapy, which specifically targets the HER2 receptor, is used to treat ERα−/PR−/HER2+ tumors and has resulted in positive survival outcomes. In contrast, the ERα−/PR−/HER2− subtype or triple negative breast cancer, which accounts for 10–17% of all breast cancer cases, and the normal breast-like (basal-like) cancer subtype, which accounts for 15% of the cases, are both without approved targeted therapies [61]. Unfortunately, drug resistance to even the most effective therapeutics is a major issue affecting breast cancer patients today. For example, although initially effective, one third of patients treated with Tamoxifen develop recurrent diseases within 15 years due to the progression of surviving tumor cells to a hormone-resistant state [62]. For these reasons, alternative drug treatments, which can be used either alone or in combination with existing therapies, are needed.

RAR-related orphan receptors (RORs), NR1F

The retinoic acid-receptor-related orphan receptor (ROR) family consists of three members: RORα (NR1F1), RORβ (NR1F2), and RORγ (NR1F3). RORs have been shown to regulate lipid and steroid metabolism, immune response and circadian rhythms. RORα is widely expressed in many tissues. RORβ is expressed in the central nervous system in particular it is localized to regions that process sensory information, such as the retina and pineal gland [63,64]. RORγ is also broadly expressed but in particular it is expressed in immune tissues such as the thymus, and is a significant factor in the development of T helper 17 cells [65]. A number of putative natural and synthetic ligands have been identified that target RORs; most notably cholesterol, cholesterol metabolites and oxysterols that have been shown to bind to the LBD of RORs and induce conformational changes that affect coregulator interactions [66,67]. While recent research has focused on the therapeutic value of inhibiting RORs for treating metabolic and autoimmune diseases, analyses of published gene expression data indicate that RORα is downregulated in multiple cancers including prostate, breast and pancreatic cancer, and suggest that RORα may act as a tumor suppressor [66,68–70].

In prostate cancer, the role of RORα as a tumor suppressor was demonstrated *in vitro* and *in vivo* using the thiazolidinedione-derivative CGP 52608 (Pubchem CID: 6509863, Table 3) [69,70]. The activation of RORα by CGP 52608 resulted in reduced cell proliferation, migration and invasiveness *in vitro,* as well as reduced growth of xenograft tumors in nude mice using the androgen-independent prostate cancer cell line, DU145 [69,70]. In addition, these studies demonstrated that activation of RORα leads to an increased expression of the cyclin-dependent kinase inhibitor p21, but decreases the expression of cyclin A. These findings, combined with the observations that there are response elements for RORα at promoter regions upstream of several cell cycle-related genes [69], suggests that RORα plays an inhibitory role in cell cycle regulation. In addition, it has recently been shown that activation of RORα, as well as RORγ, by the agonist SR1078 (Pubchem CID: 17980288, Table 3) can stabilize p53 protein a well-known tumor suppressor; an observation that further substantiates a tumor suppressing role of RORα [10,71,72]. However, further investigation is required to determine if activation of reduced protein levels of RORα in cancer would be enough to initiate adequate tumour suppression.

In breast cancer, several lines of evidence also support the role of RORα as a tumor suppressor. RORα expression is downregulated in breast cancer tumor cells compared to normal mammary tissue [73,74]. Inhibition of RORα expression results in the disruption of polarized acinar cells of mammary gland, and this disruption is known to be an early event in breast cancer development [75]. In contrast, overexpression of RORα in MCF12F breast cancer cells resulted in a reduced rate of cell proliferation [73]. RORα has been shown to act upon several pathways that are known to be involved in breast cancer development and progression. RORα binds to β-catenin that suppresses Wnt/βcatenin gene transcription, which is known to be an important pathway in cell adhesion and cell growth [76,77]. In addition, RORα has been shown to have a negative effect on the NF-κβ pathway, which is known to regulate genes important in cell proliferation, differentiation and apoptosis in breast cancer [78–81]. Additionally, RORα plays a role in steroidogenesis. In RORα knockout mice, the regulation of hydroxysteroid dehydrogenases & sulfotransferases were disrupted [82]. Furthermore, RORα has been shown to enhance the expression of aromatase in ERα+ T47D and MCF7 breast cancer cells through its binding to the aromatase promoter [83].

In addition to the discovery of compounds CGP 52608 and SR1078, there have been efforts to screen for RORα inhibitors at the Scripps Research Institute Molecular Screening Center (PubChem BioAssay AID: 610). They reported on several inhibitory compounds with IC_{50} values of <5 nM in a transcriptional activity assay (AC1M6FQE, Table 3). There are currently over 590 experimental compounds in the BindingDB that target the RORα, although none of them have been clinically approved (Fig. 1) [7]. Based on the existing knowledge on natural and synthetic ROR ligands (reviewed by Kojetin and Burris [64]) and considering the availability of a crystal structure of the ligand binding domain of RORα in complex with cholesterol (PDB ID: 1N83) [84], structural analyses of the LBS and virtual compound screening could be used to discover novel RORα agonists or antagonists and to improve the drug-like properties of existing candidates.

Liver X receptors (LXRs), NR1H

As the name suggests, liver X receptors (LXRs) were initially isolated from liver, but subsequent studies have shown that while the expression of LXRα is more restricted to certain tissues, such as the liver, kidney, and adipose, its β isoform is ubiquitously expressed in almost all tissue types (Table 1) [85]. The main functional role of LXRs is to regulate lipid homeostasis, and activation of LXRs leads to the reduction of intracellular cholesterol [86]. Since cholesterol is a precursor needed for androgen synthesis, activating LXRs can reduce androgen levels in prostate cancer cells [86].

It has been reported that a synthetic LXR agonist T0901317 (Pubchem CID: 447912, Table 3), which mimics the structure of the endogenous ligand oxysterol, was able to decrease cell proliferation of the androgen-dependent LNCaP prostate cancer cell line, as well as suppress the growth of LNCaP tumor xenografts in nude mice [85,87]. This anti-proliferative effect of the synthetic LXR agonist was deemed to be a result of increased expression of cyclin-dependent kinase inhibitor p27 and a decreased cell population in S-phase [87]. In addition, a recent study has reported that activation of LXR by agonist GW3965 (Pubchem CID: 447905, Table 3) reduced cellular proliferation and migration in LNCaP cells and reduced tumor volumes in LNCaP-xenografted nude mice, possibly through regulation of the suppressor of cytokine signaling 3 (SOCS3) pathway [88].

It should be noted, that LXRs are both anti-proliferative and lipogenic factors in breast cancer. By using a microarray gene analysis of four breast cancer cell lines, it was shown that LXR ligands disrupt cell proliferation by down-regulating genes in the E2F transcription factor family that are critical for cell cycle progression [89]. Additionally, LXR induces the expression of key regulatory genes, such as fatty acid synthase, leading to increased triglyceride production within MCF7 breast cancer cells [90]. Furthermore, targeting LXR also stimulates the efflux of cholesterol within the cell which leads to the inhibition of cell proliferation and stimulation of apoptosis in MCF7 cells [91,92]. Although the full functionality of LXRs and their ligands are yet to be fully understood, with the available evidence, LXRs could be a promising target for the treatment of breast cancer.

LXRs are one of the most challenging members of the ONR family to target on account of their essential role in hepatic lipogenesis. As a consequence there is an additional layer of complexity to the rational drug design in that the therapeutics must be tissue selective to reduce side effects. There are no approved substances reported in the DrugBank [6] despite the fact that there are over 700 experimental LXR ligands deposited in the BindingDB [7]. A number of crystal structures of the LXR α and β forms have been resolved, including LXRβ in complex with an agonist T0901317 (PDB ID: 1UPW) [93]. Both T0901317 and GW3965 are agonists for both LXRα and LXRβ. While the development of selective activators for LXRα and LXRβ has been difficult due to their highly similar amino acid sequence (74%), several recent studies have described compounds that display partial selectivity to LXRβ [94–97]. Overall, although challenging, the abundance of existing compounds and crystal structures should facilitate both ligand- and structure-based rational drug design efforts against LXRα and LXRβ.

Chicken ovalbumin upstream promoter transcription factors (COUP-TFs), NR2F

Currently, chicken ovalbumin upstream promoter transcription factor (COUP-TF) I and II, (reviewed by Lin et al. [98]) have no endogenous ligands identified. COUP-TFI and II are known to have important roles in embryonic and organ development [99]. COUP-TFs bind to DNA repeats as homo- or heterodimers [100,101]. COUP-TFs interact with the same ERα and ERβ DNA response elements, the estrogen response element (ERE); however, COUP-TFs bind to half EREs whereas ERs preferentially bind to the consensus EREs [102]. Klinge et al. demonstrated that COUP-TFs are able to directly interact with and disturb ER-DNA binding and as a consequence regulate the transcriptional activity of ERα [103]. Although the full relationship with ER is still unclear as a study by Metivier et al. demonstrated that the formation of a COUP-TF/ERα complex resulted in enhanced ERα transcriptional activity [104].

In the prostate, COUP-TFs may adopt either a tumor-promoting or tumor-suppressing role, depending on the cellular context and surrounding tumor microenvironment [99]. On one hand, it has been shown that COUP-TFII can promote prostate tumor growth by inhibiting the TGF-β-induced growth barrier and that overexpression of COUP-TFII can promote tumor metastasis when coupled with PTEN deletion [105]. On the other hand, another study reported that COUP-TFII can repress AR activation by binding to both the DBD and LBD of the AR and that overexpression of COUP-TFII reduces proliferation of LNCaP cells [106]. In addition, recent genome-wide gene expression analysis and transcription factor binding experiments revealed that COUP-TFI is a direct target gene of the AR and that its expression is downregulated upon AR binding at the promoter region [107]. This study further reported an inverse relationship between COUP-TFI gene expression and AR activation in a normal prostate development phase.

While the exact function of COUP-TFII in breast cancer remains unclear, its mRNA expression in ERα+ (MCF7, T47D, and ZR75-1) and ERα− (MDA-MB-231) breast cancer cells was shown to be significantly increased in comparison to normal epithelial cell lines [108]. Interestingly, COUP-TFII levels were reduced in Tamoxifen-resistant lines derived from MCF7 cells, and re-expression of COUP-TFII restored the growth inhibitory effect of Tamoxifen [104,109]. Further to this study, Litchfield et al. suggested that reduced expression of COUP-TFII in the endocrine resistant cell line LCC9 contributed resistance due to the suppression of NFκB activity [110].

To date, there are a number of compounds reported for COUP-TFII (but not for COUP-TFI) and no approved drugs that target the COUP-TF family. There are 231 experimental compounds with reported binding affinities toward COUP-TFII in the BindingDB deposit [7]. Among these, pyridaben (PubChem CID: 91754, Table 3) has an IC_{50} of 62 nM that was determined in a transcriptional assay by the Scripps Research Institute Molecular Screening Center (PubChem BioAssay AID: 720548). The crystal structure of the ligand binding domain of COUP-TFII is available (PDB ID: 3CJW) and shows that α-helix 10 extends into the LBD and that the AF-2 is folded into a position that blocks coregulator interactions resulting in autorepression of the receptor [111]. However, the receptor still contains a viable ligand binding pocket that when bound with ligand releases the autorepression [111]. Due to a high sequence similarity (96%) at LBD, the COUP-TFII structure can be used as a template for COUP-TFI for further *in silico* screening. In particular, *in silico* screening should be applied to discover chemical structures with high binding affinity at the LBS of COUP-TFs. Such compounds may act as either agonists or antagonists and can be used as chemical probes to further elucidate the functions of COUP-TFs in prostate and breast cancer cells.

Estrogen related receptors (ERRs), NR3B

The estrogen-related receptors (ERRs), ERRα, ERRβ and ERRγ, were so named for their sequence and structural similarity to estrogen receptors (ERs) [112]. While initially thought to share a common biological function with the ERs, ERRs do not bind to estrogens or endogenous ER ligands. There are no known endogenous ligands for ERRs, and the receptors are constitutively active

due to the structure of the LBD that leaves the AF-2 site exposed and capable of binding coregulators in the absence of ligand binding. Thus, the transcriptional properties of the ERRs are mediated by either: coactivators, such as peroxisome-proliferator activated receptor γ coactivator-1α (PGC-1α) and the p160 family of steroid receptor coactivators (SRC-1, SRC-2, SRC-3), or corepressors such as nuclear receptor interacting protein 140 (RIP140). The active ERR is able to bind to DNA at the 5′-TNAAGGTCA-3′ sequence (where N is any nucleotide) or to the classical ER response element (ERE) 5′-AGGTCANNNTGACCT-3′. ERRs regulate genes involved in the cellular energy metabolism pathways, including: glycolysis, tricarboxylic acid cycle and oxidative phosphorylation [112]. Thus, ERRα is predominantly expressed in tissues that have high energy demand, including skeletal muscle, heart, kidney, liver and adipose. Whereas, ERRβ is expressed during early embryonic development and ERRγ is expressed in the central nervous system and spinal cord.

Of the ERRs, ERRα is known to promote cell proliferation in multiple cancer cells lines, including prostate and breast cancer cells. Furthermore, inhibition of ERRα with the inverse agonist (i.e. binds to the same receptor protein but induces an opposite response to an agonist) XCT790 (Pubchem CID: 6918788, Table 3) reduces cell proliferation [113]. However, overexpression of either ERRβ or ERRγ resulted in the suppression of cell proliferation in both androgen-sensitive and androgen-insensitive prostate cancer cells, and this growth inhibition could be further enhanced by the ERRβ/γ agonist, DY131 (Pubchem CID: 5497124, Table 3) [114–116]. In addition, transcription reporter and chromatin immunoprecipitation assays demonstrated that ERRβ directly transactivates a promoter upstream of the cyclin-dependent kinase inhibitor p21 gene, which inhibits cell cycle progression [115]. While the mechanism through which the three ERR isoforms induce their effects on cell proliferation is undetermined, these findings suggest that in prostate cancer, ERRα acts as a tumor promoter, whereas ERRβ and ERRγ act as tumor suppressors.

Due to their structural similarity to ERs, ERRs (in particular ERRα and ERRγ) are most recognized for their potential function in breast cancer. Members of the ERR family can bind to EREs and affect gene expression of ER-regulated genes and may contribute to breast cancer development or progression. Interestingly, Stein et al. showed that the gene transcription products of ERRα had little overlap with those of ER [117]. Furthermore, the roles of both ERRα and ERRγ are distinct between ERα+ and ERα− breast cancer. ERRα can act as an activator or repressor of transcription in ERα− or ERα+ cells, respectively [118]. For example, ERRα can increase the expression of the estrogen-regulated trefoil factor 1 (TFF1) gene in ERα− breast tumors [119,120]. Similarly, in ERα− breast cancer samples, overexpression of ERRα induced vascular endothelial growth factor (VEGF) [117,121]. In comparison, ERRγ is predominantly associated with ERα+/PR+ breast tumors in which it promotes mesenchymal-to-epithelial transition (MET) by stimulating E-cadherin expression, which results in an inhibition of tumor growth [122,123]. Furthermore, ERRγ expression may be a marker of tamoxifen resistance and it has been shown that an AAAG tetranucleotide repeat polymorphism in untranslated region of the ERRγ gene is associated with breast cancer susceptibility [124,125]. While the exact mechanism of action of both ERRα & ERRγ in breast cancer is still not fully understood, current findings support a potential role of these receptors as therapeutic targets in breast cancer.

There are relatively few compounds reported to interact with ERRs (reviewed Lu et al. [126]). There are several crystal structures resolved for the ligand binding domains of ERRα and ERRγ. No crystal structure is available for the LBD of ERRβ, but the high protein sequence identity (80%) with ERRγ makes homology modeling feasible. However, the small size of the ligand binding pocket has made it difficult to directly target ERRs for pharmacologic activation [127]. Hence, the BindingDB catalogues only 91, 31, and 29 compounds that can bind to ERRα, β and γ, respectively [7]. The FDA-approved drugs diethylstilbestrol (DB00255, Table 3) and tamoxifen have been shown to interact with ERRs and act as antagonists [128,129]. Whereas, XCT790 (Pubchem CID: 6918788, Table 3) binds selectively to the LBD of ERRα and has an IC_{50} of 0.37 μM [130]. Methods that combined structure-based virtual screening and biological functional assays have identified several isoflavones including Genistein that bind at the LBS of the ERRs [29]. SR16388 (Pubchem CID: 54612678, Table 3), a novel steroidal antiestrogen, disrupts the interaction between ERRα and its coactivator peroxisome-proliferator activated receptor γ coactivator-1α to inhibit ERRα activity. SR16388 has an IC_{50} of 0.2 μM on malignant cell growth in xenograft models and has successfully completed phase I and phase II clinical trials [131].

Nerve growth factor 1B-like (NGFIB, NURR1, NOR1), NR4A

The nerve growth factor 1B-like family is comprised of three members: NGFIB (NUR77 or NR4A1), NURR1 (NR4A2), and NOR1 (NR4A3). These nuclear receptors are unique due to the fact that they lack the LBS within their LBD [45]. Members of this family regulate a wide range of biological processes including metabolism, inflammation, vascular function, steroidogenesis, and functions of the central nervous system [132,133]. NIGFB is known to interact with specific response elements 5′-AAAGGTCA-3′ in the form of a monomer or homodimer [134,135]. The physiological role of NGFIB is not yet fully understood, but several studies indicate it has a role in cancer cell death and survival [136–139]. Interestingly, NGFIB is found to have pro-survival benefits while in the nucleus, but promote apoptosis through Bcl-2/cytochrome c pathway when translocated into mitochondria [140,141]. NGFIB has been previously shown to exhibit pro-oncogenic activity in several cancers, including cervical, lung, colon and pancreatic cancers. A number of compounds, including 1,1-bis(3′-indolyl-1-(p-substituted phenyl)methanes (C-DIM; Pubchem CID: 54612678, Table 3), which can activate or deactivate NGFIB, have been reviewed [133]. In addition, cytosporone B has been previously reported as a naturally occurring agonist for NGFIB and was capable of promoting apoptosis and reducing xenograft tumor growth by inducing NGFIB expression and translocation to mitochondria [142].

With regard to prostate cancer, a recently reported immunohistochemistry experiment revealed that expression of NURR1 protein is higher in prostate cancer tissues compared to benign ones [143]. Furthermore, this NURR1 expression was positively correlated with several clinical indicators, including tumor stage and Gleason score. Knockdown of NURR1 protein with siRNA resulted in reduced proliferation, migration and invasion as well as induced apoptosis in prostate cancer cells. Hence, the study suggested that NURR1 may be used as a biomarker and therapeutic target in prostate cancer [143].

In breast cancer, various diindolylmethanes (DIMs) have demonstrated agonistic effects toward the NGFIB [144]. Chinthalapalli et al. demonstrated that in the presence of DIM, MCF7 breast cancer cells that express NGFIB have a reduction in their proliferation capacity and increased apoptosis [145]. In addition, NGFIB is known to regulate and affect transcriptional responses in other ONRs and NRs, such as COUP-TFs and retinoid X receptors (RXRs). Furthermore, it has been demonstrated that NGFIB is capable of producing a heterodimer with RXR that induces apoptosis and growth inhibition in MDA-MB-231 breast cancer cells through activation of RARα and subsequent pathways [146].

There is not yet an approved drug targeting any member of the NR4A subfamily. The recent research efforts dedicated toward the NGFIB have generated 281 experimental compounds targeting this

protein. There are currently two crystal structures available for NGFIB (PDB ID: 2QW4 and 3V3E [147]), which should further aid in the identification or modification of new or existing compounds. In contrast, there are only 12 compounds which have been shown to interact with NURR1. The crystal structure of the NURR1 LBD (PDB ID: 1OVL [45]) is observed to be filled by hydrophobic substituents, which suggests that it may be a true ONR without a ligand [45]. Interestingly, 6-mercaptopurine and a few structural analogues have been shown to activate the NR4A subfamily. The action of 6-mercaptopurine occurs through the NTD in an AF-1 dependent manner [148]. The NTD of NRs is traditionally thought of as being structurally unstable and thus has not been the focus of many drug targeting approaches. However, given the identification of NTD directed compounds, further work in this direction may yield more promising results that could be applied to other ONR and NR targets.

DAX1, NR0B1

While the physiological significance of DAX1 in females is unclear, knockout mouse studies have demonstrated that DAX1 is essential for the maintenance of male reproductive functions [149]. Furthermore, DAX1 is uniquely and highly expressed in steroidogenic tissues [150]. For example, DAX1 is known to be a negative regulator of aromatase mRNA expression in ovarian cells that are the sites of androgen and estrogen production [151]. Additionally, several studies suggest that the primary function of DAX1 is as a coregulator protein, since it is capable of affecting the activity of several receptors, including an ONR, steroidogenic factor-1 (SF-1), as well as, ER, PR and AR [152].

While not detected in normal prostate tissue, DAX1 is expressed in prostate cancer epithelium, and its expression is inversely correlated with Gleason score [153]. DAX1 has been shown to directly interact with the AR, inhibiting its transcriptional activity through disruption of the AR N- and C-terminal interaction [154]. DAX1, which is able to cycle between the cytoplasm and the nucleus, is also capable of tethering the AR in the cytoplasm or nucleus of the cell, further restricting AR transcriptional activity [154].

A recent publication by Conde et al. established that while DAX1 is expressed in the benign form of breast carcinoma (*in situ*), it is more significantly expressed in invasive breast cancer [155]. There was also a report of a direct correlation between lymph node metastasis and the expression of DAX1 protein. In addition, it was observed that DAX1 has also been shown to directly interact with AR, ERα and ERβ in breast cells, but has only been shown to influence the intracellular positioning of AR and ER-β, and not ER-α [155,156]. Thus, DAX1 may be a novel coregulator of nuclear receptor action in both prostate and breast tissues.

DAX1 is unique among other members of the NR family in that it lacks a classical DBD domain and it has an N-terminal domain that contains three repeats of a unique 70 amino acid motif [157]. Additionally, DAX1 contains a potent transcriptional repressor domain in its C-terminus [158,159]. Although there is not yet a protein structure available for human DAX1, the LBD of the mouse homolog has been resolved (PDB ID: 3F5C [160]) and established that the C-terminal domain does not contain a ligand binding site. The structure of the human domain shares 68% identity with the mouse so it is possible that the human DAX1 LBD surface may contain a pocket that could be targeted by small molecules. Additionally, drugs may also be developed that target alternative surfaces of DAX1, such as areas involved in coregulator interactions or the N-terminal domain because of its unique sequence repeat. Currently, there are over 200 compounds indicated in BindingDB to bind to DAX1 [7]. The Scripps Research Institute Molecular Screening Center (PubChem BioAssay AID: 687017) reported that compound thiocolciran (Pubchem CID: 426728, Table 3) demonstrates an IC_{50} of 212 nM in a luminescence-based high-throughput cell screening assay. Additionally, there are two FDA-approved drugs that have been shown to indirectly target DAX1 [6]. One is dexamethasone, a potent glucocorticoid, and the other is Tretinoin, a naturally occurring derivative of vitamin A. Thus, DAX1 is a promising target due to its unique structure that may provide an alternative strategy for drug targeting.

Conclusions

In recent decades nuclear receptors have been actively exploited as drug targets mainly due to their regulatory roles in numerous cellular processes and the presence of "druggable" ligand binding domain. Unlike most other transcriptional factors that lack a well-defined active site, the function of NRs can be modulated by small molecules that compete with endogenous ligands to act as either antagonists or agonists. Within the NR family, hormone receptors such as the AR and ER have been studied intensively, and represent established targets for a number of approved drugs in prostate and breast cancers. However for both cancers, current hormonal therapies often lead to the development of drug resistance; thus, targeting alternative proteins and pathways may provide a way to treat resistant diseases. Given the recent advances in computational modeling techniques and the availability of protein structures, ONRs present an exciting opportunity for drug development. As described in this review, with the aid of *in silico* modeling much progress has been made on the identification of natural and synthetic ONR ligands. Furthermore the recent successes targeting alternative protein–protein interaction sites such as the AF-1, AF-2, and BF-3 site of the AR highlight the possibilities for targeting these sites on ONRs. Ultimately, identification of novel small molecules that bind to ONRs will provide a better understanding of their biology, structure and interactions. In addition, this accumulated knowledge on the endogenous functions of ONRs will provide a solid foundation for future drug discovery with therapeutic values that may go beyond prostate and breast cancers.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgements

The authors wish to thank Dr. Amy Lubik for her editorial comments towards this manuscript. This work was supported by an operating Grant 272111 from Canadian Institutes of Health Research, a Movember Discovery Program award, and Prostate Cancer Canada with generous support from Canada Safeway – Grant SP2013-02.

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